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Transcriptional Regulation by the SACCHAROMYCES CEREVISIAE Centromere-Binding Protein CP1: a Dissertation

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TRANSCRIPTIONAL REGULATION BY THE *SACCHAROMYCES CEREVISIAE* CENTROMERE-BINDING PROTEIN CP1

A Dissertation Presented

By

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To my wife Laurie who by way of love, support, and encouragement helped me through 6 years of graduate school and to my parents John and Inez without whose love and support I would not have made it this far.

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ABSTRACT

CP1 (encoded by the gene *CEP1*) is a sequence-specific DNA-binding protein of *Saccharomyces cerevisiae* that recognizes a sequence element (CDEI) found in both yeast centromeres and gene promoters. Strains lacking CP1 are viable but exhibit defects in growth, chromosome segregation, and methionine biosynthesis. To investigate the basis of the methionine requirement, a YEp24-based yeast genomic DNA library was screened for plasmids which suppressed the methionine auxotrophy of a *cep1* null mutant. The suppressing plasmids contained either *CEP1* or DNA derived from the *PHO4* locus. PHO4 encodes a factor which positively regulates transcription of genes involved in phosphate metabolism via an interaction with CDEI-like elements within the promoters of these genes. Subcloning experiments confirmed that suppression correlated with increased dosage of *PHO4*. *PHO4^c*, *pho80*, and *pho84* mutations, all of which lead to constitutive activation of the PHO4 transcription factor, also suppressed *cep1* methionine auxotrophy. The suppression appeared to be a direct effect of PHO4, not a secondary effect of *PHO* regulon derepression, and was dependent on a second transcriptional regulatory protein encoded by *PHO2*. Spontaneously arising extragenic suppressors of the *cep1* methionine auxotrophy were also isolated; approximately one-third of the them were alleles of *pho80*. While *PHO4* overexpression suppressed the methionine auxotrophy of a *cep1* mutant, *CEP1* overexpression failed to suppress the phenotype of a *pho4* mutant; however, a *cep1* null mutation suppressed the low-P_i growth deficiency of a *pho84* mutant. The results suggest that CP1 functions as a transcriptional regulator of *MET* genes,

and that activation of PHO4 restores expression to those genes transcriptionally-disabled by the *cep1* mutation. The results also suggest the existence of a network that cross-regulates transcription of genes involved in methionine biosynthesis and phosphate metabolism.

A direct molecular approach to investigate CP1's role in *MET* gene expression was also taken. CDEI sites are associated with the promoter regions of most *MET* genes, but only *MET16*, the gene encoding PAPS reductase, has been shown to require CP1 for expression; both PAPS reductase activity, and *MET16* mRNA are absent in *cep1* mutants. Results of the present study demonstrate that CP1 participates in two systems which regulate expression of *MET16*, one triggered by methionine starvation and requiring the transactivator MET4 (pathway-specific control), and the other triggered by starvation for many different amino acids and requiring GCN4 (general control). CP1 was shown to mediate its regulatory function through the upstream CDEI site, and to act directly or indirectly to modulate the chromatin structure of the *MET16* promoter. In addition, the *pho80* mutation was found to partially restore *MET16* expression to the *cep1* strain, confirming the proposed nature of *PHO4* suppression. A second methionine biosynthetic gene *MET25*, was also analyzed. Like *MET16*, *MET25* was found to be regulated by both pathway-specific and general control mechanisms, but in contrast to *MET16*, CP1 only participated in the pathway-specific response of this gene. The results demonstrate that CP1, possibly by modulating changes in chromatin structure, assists the regulatory proteins MET4 and GCN4 in activating transcription of *MET* genes.

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CHAPTER I

INTRODUCTION

The centromere is the physical locus on each chromosome which nucleates the formation of a complex of proteins, collectively termed the kinetochore. During mitotic and meiotic divisions, the centromere and its associated kinetochore execute several functions essential for the high fidelity with which chromosomes are segregated: these include sister chromatid cohesion, attachment of the chromosome to the spindle microtubules and generation of the mechanochemical forces necessary for segregation (reviewed by Willard 1990). In higher eucaryotes, the kinetochore appears by electron microscopy as a complex trilaminar structure (Rattner 1991). In contrast, kinetochores of the yeast *Saccharomyces cerevisiae* are not visible by electron microscopy; a single microtubule appears to attach directly to each centromere (Peterson and Ris 1976). Failure to detect a kinetochore by electron microscopy probably reflects the structural simplicity of this organelle in yeast. The functional *S. cerevisiae* centromere is also simple in structure, being only 125 bp in length (Cottarel *et al.* 1989; Fitzgerald-Hayes, Clarke and Carbon 1982; Hieter *et al.* 1985), and organized into three conserved centromere DNA elements (CDE's) (reviewed by Fitzgerald-Hayes 1987). The left border is formed by CDEI, 8 bp in length with the consensus sequence PuTCACPuTG (where Pu=purine), and the right border by CDEIII, a conserved 25-bp sequence with partially dyad symmetry. The central element CDEII, consists of 78-86-bp of AT-rich DNA. In terms of function, CDEIII is essential while CDEI and CDEII play less important roles (Cumberledge and Carbon 1987; Gaudet and Fitzgerald-Hayes 1987; Hegemann *et al.* 1988; McGrew, Diehl and Fitzgerald-Hayes 1986; Panzeri *et al.* 1985).

Proteins which bind specifically to yeast centromeres have been puri-

fied and characterized. Bram and Kornberg (1987) were first to describe a partially purified activity which bound specifically to CDEI. This same protein (CP1/CPF1/CBP1/CBF1) was subsequently purified to homogeneity by three groups (Baker, Fitzgerald-Hayes and O'Brien 1989; Cai and Davis 1989; Jiang and Philippsen 1989). CP1 (as it shall be refered to here) is comprised of a single 39.4 kDa polypeptide, binds its recognition site as a homodimer and is involved in maintaining chromatid-kinetochore adhesion (Masison and Baker 1992). A protein complex which binds specifically to CDEIII has also been described (Lechner and Carbon 1991). This complex is composed of at least three polypeptides, CBF3A (110 kd), CBF3B (64 kd), and CBF3C (58 kd), and has been shown to possess a minus-end-directed microtubule motor activity (Hyman *et al.* 1992).

Although the function of the CBF3 complex is most likely limited to the centromere, the function of CP1 is not. Bram and Kornberg (1987) initially proposed the idea of a noncentromeric role for CP1 based on three observations. First, the abundance of CP1 (>500 copies/cell) was deemed to be much greater than would be expected if its activity was restricted to centromeres. In fact, the CBF3 protein complex appears to be present in the cell at only 1 copy per centromere (16 copies per haploid cell), far below the level of CP1 (Lechner and Carbon 1991). Second, a computer-assisted search of a DNA sequence database revealed that CDEI sites were not restricted to centromeres, but distributed throughout the yeast genome, including the 5'-flanking regions of the *GAL2* and *TRP1* genes. Third, CP1 was found to be the only protein in yeast cell extracts capable of binding these noncentromeric CDEI sites.

The gene encoding CP1 was cloned independently by three groups and

designated *CEP1* (Baker and Masison 1990), *CBF1* (Cai and Davis 1990), and *CPF1* (Mellor *et al.* 1990). Sequence analysis revealed that CP1 belonged to the helix-loop-helix (HLH) family of DNA-binding proteins (Cai and Davis 1990). All members of this family share a region of homology spanning 60 amino acid residues and predicted to form two amphipathic helices separated by a loop (Murre, McCaw and Baltimore 1989). Not all HLH proteins bind DNA, however, those that do bind DNA contain a region rich in basic amino acid residues (the b-HLH proteins) immediately preceding the HLH domain (Davis *et al.* 1990). Consistent with CP1's assignment to this family, the CDEI site contains the core consensus sequence CANNTG (where N=any residue), recognized by all DNA-binding b-HLH proteins (Cai and Davis 1990; Lassar *et al.* 1989). Disruption of *CEP1* was found to be nonlethal and confirmed the multifunctional nature of CP1. Strains lacking a functional *CEP1* gene product displayed an array of phenotypes including an increased frequency of mitotic and meiotic chromosome missegregation, slow growth, and a requirement for methionine (Baker and Masison 1990; Cai and Davis 1990; Masison and Baker 1992; Mellor *et al.* 1990).

Several studies have indicated that both CP1 and an intact CDEI site are required for optimal centromere function. Mutation or deletion of CDEI from the centromere results in 3- to 70-fold increases in mitotic chromosome loss rates (Cumberledge and Carbon 1987; Gaudet and Fitzgerald-Hayes 1989; Hegemann *et al.* 1988), and the magnitude of the effect is correlated with decreased CP1 binding affinity (Baker, Fitzgerald-Hayes and O'Brien 1989; Cai and Davis 1989). The trans mutation, *i.e.*, disrupting *CEP1*, has a quantitatively similar consequence; the mitotic chromosome loss rate is increased 9-

to 25-fold (Baker and Masison 1990; Cai and Davis 1990). When the effects of cis and trans mutation are compared directly, they are found to be equivalent and nonadditive (Baker and Masison 1990). Biochemical experiments have shown that CDEI sites are protein-bound *in vivo* and that CP1 is required to maintain normal chromatin structure in the CDEI region (Densmore, Payne and Fitzgerald-Hayes 1991; Mellor *et al.* 1990). Taken together, these results indicate that the role of CDEI in the assembly and/or function of the *S. cerevisiae* centromere (kinetochore) is mediated through CP1 and that lack of CP1 interaction at centromeric CDEI sites accounts for the chromosome loss phenotype of *cep1* null mutants.

The requirement for CP1 in methionine metabolism is probably exerted at the level of transcription. Almost every methionine biosynthetic (*MET*) gene sequenced to date contains at least one CDEI site in its 5'-flanking region. These genes include, *MET2*, *MET3*, *MET4*, *MET8*, *MET14*, *MET16*, *MET22*, *MET25*, and *SAM2* which encodes S-adenosylmethionine synthetase (Cherest, Thomas and Surdin-Kerjan 1990; Gläser *et al.* 1993; Korch, Mountain and Byström 1991; Thomas, Barbey and Surdin-Kerjan 1990; Thomas, Cherest and Surdin-Kerjan 1989; Thomas, Jacquemin and Surdin-Kerjan 1992). Transcription of many (if not all) of these genes is regulated in response to methionine availability (Cherest, Thao and Surdin-Kerjan 1985; Mountain *et al.* 1991; Thomas, Barbey and Surdin-Kerjan 1990; Thomas, Cherest and Surdin-Kerjan 1989). In the presence of sufficient levels of methionine, transcription is repressed via a mechanism which senses an elevated concentration of S-adenosylmethionine (AdoMet), the end product of the methionine biosynthetic pathway (Thomas, Cherest and Surdin-Kerjan

1989). Upon methionine starvation, AdoMet-mediated repression is relieved and transcription is activated by a mechanism which depends upon a positive regulator encoded by the *MET4* gene (Thomas, Jacquemin and Surdin-Kerjan 1992). For the *MET25* gene (encoding O-acetylhomoserine sulfhydrylase), transcriptional activation also requires two upstream CDEI sites (Thomas, Cherest and Surdin-Kerjan 1989); promoter deletions that encompass both CDEI sites reduce message levels to 10% of the wild-type level.

Although the CDEI sites appear to be required for transcriptional activation of *MET* genes in response to methionine limitation, the role of CP1 in this response is not so clear. While Mellor *et al.* (1991) have reported that the *cep1* mutation has no effect on *MET25* message levels, Thomas, Jacquemin and Surdin-Kerjan (1992) have reported a 3-fold reduction in *MET25* message levels in the *cep1* mutant. Furthermore, the message levels of *MET2*, *MET3*, and *SAM2*, all of which possess upstream CDEI sites, appear normal in the *cep1* strain (R. Baker, unpublished observations). Recently, a study has shown that *cep1* strains lack at least two activities essential for methionine biosynthesis: 3'-phosphoadenylylsulfate (PAPS) reductase and sulfate permease (Thomas, Jacquemin and Surdin-Kerjan 1992). The lack of PAPS reductase activity can be traced to a defect in expression of *MET16*, the gene encoding this activity; when grown under derepressing conditions, *cep1* strains fail to accumulate detectable levels of *MET16* message (Thomas, Jacquemin and Surdin-Kerjan 1992). Presumably, the lack of *MET16* mRNA in the *cep1* mutant is due to a block in transcriptional activation, but evidence to prove CP1 functions at this level has not been obtained.

A transcriptional role for CP1 would certainly be consistent with its as

signment to the HLH family. Most, if not all, of the established HLH family members are known or suspected to be involved in transcriptional regulation (*e.g.*, MyoD, *myc*, *daughterless*, E12/E47, AP-4, USF) (Gregor, Sawadogo and Roeder 1990; Hu *et al.* 1990; Sun and Baltimore 1991). *S. cerevisiae* is known to contain other HLH factors, and one of them, the product of the gene *PHO4*, contains an HLH-adjacent basic (DNA-binding) region highly similar to CP1 (Berben *et al.* 1990; Dang *et al.* 1992; Fisher, Jayaraman and Goding 1991; Mellor *et al.* 1990). PHO4 protein is a positive activator of genes involved in phosphate metabolism (*PHO* genes) and appears to be a transcription factor in the conventional sense, *i.e.*, it binds to its cognate site in DNA and interacting directly or indirectly with RNA polymerase II, stimulates transcription initiation (Hayashi and Oshima 1991; Ogawa and Oshima 1990; Vogel, Hörz and Hinnen 1989).

If CP1 does regulate *MET16* at the level of transcription, it probably does so in a way fundamentally different from that by which PHO4 regulates expression of *PHO* genes. Unlike PHO4, CP1 does not appear to contain an autonomous transactivation function; LexA-CP1 or GAL4 DNA-binding domain-CP1 fusion proteins fail to activate LexA or GAL4 DNA binding site-driven reporter genes, respectively (Thomas, Jacquemin and Surdin-Kerjan 1992; R. Baker unpublished data). Furthermore, CDEI sites do not exhibit UAS activity when placed alone upstream of a reporter gene (Buchman and Kornberg 1990; Thomas, Jacquemin and Surdin-Kerjan 1992). However, a CDEI site in combination with an adjacent 9-bp element from the *MET25* promoter produces a strong methionine-responsive UAS activity that is entirely dependent on both CP1 and *MET4* (Thomas, Jacquemin and Surdin-

Kerjan 1992). The strong CP1-dependence of this construct stands in contrast to the fairly weak effect of the *cep1* mutation on expression of the endogenous *MET25* gene. Taken together, the results suggest that for the minimal *MET25* promoter, CP1 provides an essential "accessory" function, the loss of which can be compensated for within the context of the native promoter.

One mechanism by which CP1 might act would be the recruitment of other factors (such as *MET4*) to the *MET16* promoter, similar to the role proposed for RAP1 in *HIS4* expression (Devlin *et al.* 1991). Like CP1, RAP1 only activates transcription efficiently in combination with other factors (Buchman and Kornberg 1990), and at the *HIS4* promoter, is required for activation by both a general control and a basal control system. Alternatively, CP1 may act independently of *MET4*, providing some other essential function. Regardless of which model is correct, both require sequence-specific DNA binding by CP1 as a means of targeting activity to the *MET16* promoter. However, one group has reported that a DNA-binding deficient form of CP1 is still capable of maintaining methionine-independent growth, despite being unable to provide optimal centromere function (Mellor *et al.* 1991). This finding has led to the suggestion that DNA binding is not essential for CP1's role in methionine biosynthesis. Since other work has shown that greatly diminished expression of *CEP1* can lead to the same phenotype—loss of the centromere function without the accompanying methionine auxotrophy (Masison, O'Connell and Baker 1993)—it may be that CP1 is in functional excess over what is needed to maintain methionine prototrophy and that a drastic reduction in either DNA-binding activity or expression of the protein will still allow cells to grow in the absence of methionine.

Evidence to indicate that DNA binding is essential for the function of CP1 in methionine biosynthesis was also provided by an elegant series of domain-swap experiments. Dang *et al.* (1992) constructed several chimeric versions of CP1 by removing the basic (DNA-binding) domain of CP1 and replacing it with the homologous region from one of three b-HLH proteins. These chimeras were tested for the ability to rescue the methionine requirement of a *cep1* strain. Methionine-independent growth was maintained if the basic domain of CP1 was replaced with the basic domain of either c-Myc or USF, two b-HLH proteins which normally recognize the CDEI core sequence CACGTG. However, a CP1 chimera containing the basic domain of AP4 was unable to rescue methionine prototrophy, presumably because AP4 normally recognizes CAGCTG. Replacement of a methionine residue in the noninterchangeable AP4 basic domain with an arginine—arginine is conserved at this position in b-HLH proteins which recognize CACGTG—allowed recognition of CACGTG, and restored methionine-independent growth. These results argue that like CP1's role at the centromere, its role in methionine biosynthesis requires specific recognition of CACGTG.

The apparent multifunctionality of CP1 is not unique to this protein, but is the hallmark of a family of yeast sequence-specific DNA-binding proteins known as general regulatory factors (GRFs). Although structurally dissimilar, all of these factors are moderately abundant proteins with many genomic sites of interaction and are involved in diverse chromosome-related processes. Members of this family include CP1, RAP1, REB1, and ABF1, and with the exception of CP1, all provide essential functions. GRF's have been shown to be associated with chromosome origins of replication (ABF1),

telomeres (RAP1), and centromeres (CP1). In addition to their association with genetic loci involved in chromosome maintenance, all have been implicated as positive and negative regulators of transcription (Buchman and Kornberg 1990; Chasman *et al.* 1990; deWinde and Grivell 1992; Kimmerley *et al.* 1988; Kurtz and Shore 1991; Sussel and Shore 1991; Thomas, Jacquemin and Surdin-Kerjan 1992). Each of these factors has been implicated to some degree in modulating chromatin structure, but it is presently not known if GRF's are truly multifunctional or if each factor carries out the same or similar function at all of its recognition sites.

The aim of my thesis research was to characterize the methionine auxotrophy of *cep1* null mutants. This study was initiated using two approaches to isolate suppressors of the *cep1*-associated methionine auxotrophy (O'Connell and Baker 1992). In one case, a yeast multicopy plasmid gene bank was screened for plasmids which would rescue methionine prototrophy, and in the other, spontaneously-arising Met^+ extragenic suppressors were isolated. Rather than leading to one or more *MET* genes, both lines of investigation led to genes regulating phosphate metabolism; suppressing plasmids contained DNA from the *PHO4* locus, and one third of the extragenic suppressors represented loss-of-function alleles of the negative regulatory gene *PHO80*. With respect to suppression, epistasis studies revealed that *PHO4* encoded the most direct-acting component, and that suppression also required a second regulatory factor encoded by the *PHO2* gene. Additionally, I found that the *cep1* mutation adversely affected expression of acid phosphatase activity and suppressed the phenotype of a phosphate permease mutant. These results demonstrate that the transcription factor encoded by *PHO4* can functionally

substitute for CP1 to maintain methionine-independent growth, and suggest the existence of a network which cross-regulates expression of *MET* and *PHO* genes.

The second half of this thesis describes a molecular approach to investigate the role of CP1 in the regulated expression of methionine biosynthetic genes. A systematic study of *MET16* revealed that transcription of this gene can normally be activated through two partially independent mechanisms, one of which is triggered by methionine starvation and requires the transactivator *MET4* (pathway-specific control), and one of which is triggered by starvation for many different amino acids and requires GCN4 (general control). The latter response serves to coordinate the expression of many amino acid biosynthetic genes in yeast and has been termed the general control response. Most importantly, I found that CP1 was required for either pathway to operate properly and mediated its function through the *MET16* CDEI site. The results also explain why *MET16* appears unique among *MET* genes in its transcriptional requirement for CP1. As a first step towards understanding the mechanism by which CP1 acts, I analyzed the chromatin structure of the *MET16* locus in both a wild-type and a *cep1* strain. The results suggest that CP1 functions to modulate chromatin structure, but in a manner distinct from that proposed for other general regulatory factors.

CHAPTER II

MATERIALS AND METHODS

Strains, media, and general methods. Yeast strains used in this study are listed in Table 1. Strain YPH98 (Spencer *et al.* 1990) was obtained from P. Hieter, strains NBW7, NBD4-1, and NBD82-1 (Ogawa and Oshima 1990) from Y. Oshima, strains h-A and 5-43 from L. Bergman, strains F113 and F212 from A. Hinnebush, and strain CD107 from Y. Surdin-Kerjan. The *pho3* and *pho5* mutations present in diploid K52 originated from strain GG100-14D (Bergman 1986) obtained from D. Tipper. All other strains were constructed by myself using standard genetic methods. Yeast transformations were performed by the lithium acetate procedure (Ito *et al.* 1983) as modified by Schiestl and Gietz (1989). *E. coli* strain RR1 was the host for isolating and maintaining all plasmids. Plasmids were rescued from yeast transformants using a modification of the procedure of Birnboim and Doly (1979) as follows: Cells from 1.5 ml of a selectively grown culture were pelleted and resuspended in 100 µl of 1.2 M sorbitol-0.12 M K_2HPO_4-0.033 M citric acid (pH 5.9) containing 2.5 mg/ml Zymolyase-100T. After incubating 10-30 min to obtain spheroplasts, the Birnboim and Doly procedure was followed from the alkaline lysis step.

Media were as described (Baker and Masison 1990) except for P_i-depleted YEPD which was prepared as described by Rubin (1974) and adjusted to pH 4.7. Synthetic media used for growing cells for acid phosphatase assays contained 0.17% P_i-depleted yeast nitrogen base (lacking amino acids and ammonium sulfate), 25 mM sodium citrate (pH 4.7), and 2% glucose. Amino acids (40 *u*g/ml), adenine (20 *u*g/ml) and uracil (20 *u*g/ml) were added as needed. High phosphate, 3/5 phosphate, and low phosphate media contained

TABLE 1

Strains

Strain	[a] Genotype
D1-1C	*MATα cry1 his4-580 lys2 trp1 SUP4-3 ade2-1 leu2 ura3 ade3 cep1::URA3-11*
D1-6C	*MATα cry1 his4-580 lys2 trp1 SUP4-3 ade2-1 leu2 ura3 ade3*
SMAF13α	*MATα cry1 his4-580 lys2 trp1 SUP4-3 ade2-1 leu2 ura3 ade3 cep1::URA3-11 sma1-F13*
R31-3BR	*MATα leu2Δ1 lys2-801 trp1Δ1 ura3-52 ade2-101 his3Δ200 cep1::TRP1*
R31-5C	*MAT***a** *leu2Δ1 lys2-801 trp1Δ1 ura3-52 ade2-101 his3Δ200 cep1::TRP1* CFVII (*RAD2*.d.*URA3.SUP11*)
R31-1A	*MATα leu2Δ1 lys2-801 trp1Δ1 ura3-52 ade2-101 his3Δ200* CFVII (*RAD2*.d.*URA3.SUP11*)
h-A	*MATα leu2Δ1 lys2-801 trp1Δ1 ura3-52 ade2-101 his3Δ200 pho80::LEU2*
5-43	*MATα leu2Δ1 lys2-801 trp1Δ1 ura3-52 ade2-101 his3Δ200 pho2::LEU2*
NBD82-1	*MAT***a** *leu2-3,112 pho3-1 trp1-289 ura3-1,2 can1 PHO4^{c}-1 ade2 his3-532*
K22-T8	*MAT***a** *leu2-3,112 pho3-1 trp1-289 ura3-1,2 can1 PHO4^{c}-1 ade2 his3-532 cep1::TRP1*
NBD4-1	*MAT***a** *leu2-3,112 pho3-1 trp1-289 ura3-1,2 can1 ade2 his3-532 pho4::HIS3*

TABLE 1 cont'd

NBW7	*MAT***a** *leu2-3,112 pho3-1 trp1-289 ura3-1,2 can1 ade2 his3-532*
K43-T1	*MAT***a** *leu2-3,112 pho3-1 trp1-289 ura3-1,2 can1 ade2 his3-532 cep1::TRP1*
K6	*MAT***a**/*MATα cry1/cry1 HIS4/his4-580 lys2/lys2 SUP4-3/SUP4-3 ade2-1/ade2-1 leu2/leu2 ura3-52/ura3-52 ade3/ade3 trp1::LEU2/TRP1 can1/CAN1 TYR1/tyr1 cep1::ura3/cep1::URA3-11 cyh2/CYH2 SMA1/sma1-F13*
K23	*MAT***a**/*MATα leu2Δ1/leu2Δ1 lys2-801/lys2-801 trp1Δ1/trp1Δ1 ura3-52/ura3-52 ade2-101/ade2-101 his3Δ200/his3Δ200 cep1::TRP1/CEP1 pho80::LEU2/PHO80* CFVII (*RAD2*.d.*URA3.SUP11*)
R33R63	*MAT***a**/*MATα leu2Δ1/leu2Δ1 lys2-801/lys2-801 trp1Δ1/trp1Δ1 ura3-52/ura3-52 ade2-101/ade2-101 his3Δ200/HIS3 cep1::TRP1/cep1::TRP1*
K37	*MAT***a**/*MATα leu2Δ1/leu2Δ1 lys2-801/lys2-801 trp1Δ1/trp1Δ1 ura3-52/ura3-52 ade2-101/ade2-101 his3Δ200/his3Δ200 cep1::TRP1/cep1::TRP1 sma1-1c/SMA1 CYH2/cyh2 PHO81/pho81::HIS3* CFVII (*RAD2*.d.*URA3.SUP11*)
K39	*MAT***a**/*MATα leu2Δ1/leu2Δ1 lys2-801/lys2-801 trp1Δ1/trp1Δ1 ura3-52/ura3-52 ade2-101/ade2-101 his3Δ200/his3Δ200 cep1::TRP1/cep1::TRP1 PHO2/pho2::LEU2 PHO80/pho80::LEU2* CFVII (*RAD2*.d.*URA3.SUP11*)
K45	*MAT***a**/*MATα leu2Δ1/leu2Δ1 lys2-801/lys2-801 trp1Δ1/trp1Δ1 ura3-52/ura3-52 ade2-101/ade2-101 his3Δ200/his3Δ200 cep1::TRP1/CEP1 pho80::LEU2/PHO80 PHO84/pho84::URA3*

TABLE 1 cont'd

K47	*MAT***a**/*MATα cry1/CRY1 HIS3/his3Δ200 his4-580/HIS4 lys2/lys2-801 TRP1/trp1Δ1 SUP4-3/sup4⁺ ade2-1/ade2-101 leu2/leu2Δ1 ura3-52/ura3-52 can1/CAN1 ade3/ADE3 cep1::URA3-11/cep1::TRP1 PHO80/pho80::LEU2 sma1-F13/SMA1*
K52	*MAT***a**/*MATα leu2Δ1/leu2Δ1 lys2-801/lys2-801 trp1Δ1/trp1 ura3-52/ura3-52 ade2-101/ADE2 his3Δ200/his3 cep1::TRP1/CEP1 pho80::LEU2/PHO80 pho3pho5/PHO3PHO5* CFVII (*RAD2*.d.*URA3.SUP11*)
K23-9A	*MAT***a** *leu2Δ1 lys2-801 trp1Δ1 ura3-52 ade2-101 his3Δ200*
K23-9B	*MATα leu2Δ1 lys2-801 trp1Δ1 ura3-52 ade2-101 his3Δ200 cep1::TRP1*
K23-9D	*MAT***a** *leu2Δ1 lys2-801 trp1Δ1 ura3-52 ade2-101 his3Δ200 cep1::TRP1 pho80::LEU2*
K55-M47	*MAT***a** *leu2Δ1 lys2-801 trp1Δ1 ura3-52 ade2-101 his3Δ200 met16-47*
K56-M48	*MAT***a** *leu2Δ1 lys2-801 trp1Δ1 ura3-52 ade2-101 his3Δ200 met16-48*
CD107	*MAT trp1 met4::TRP1*
F113	*MAT***a** *ino1 ura3-52*
F212	*MAT***a** *ino1 ura3-52 gcn4-Δ1*
K64-T1	*MAT***a** *ino1 ura3-52 cep1::URA3-10*
K63-T1	*MAT***a** *ino1 ura3-52 gcn4-Δ1 cep1::URA3-10*

1,500 mg, 900 mg, and 20 mg KH_2PO_4 per liter, respectively. In addition, KCl was added to 3/5 phosphate, and low phosphate media at 600 mg and 1,500 mg per liter, respectively. Inorganic phosphate was depleted from yeast nitrogen base by precipitation as $MgNH_4PO_4$ as follows: For a 10X stock solution, 8.5 g of yeast nitrogen base was dissolved in 400 ml water. Fifty milliliters each of 1 M $MgSO_4$ and concentrated NH_4OH were added and the solution stirred at room temperature for 30 min. The precipitate was removed by filtering the solution two successive times through Whatman No. 1 filter paper, and the filtrate was adjusted to pH 4.7 with concentrated HCl. DNA sequencing was carried out using the CircumVent Thermal Cycle Sequencing Kit (New England Biolabs, Inc.) as instructed by the manufacturer, and β-galactosidase activity was assayed in whole yeast cells as described (Rose, Winston and Hieter 1990).

Plasmids. Plasmids containing the various *PHO4* alleles (pAC331, pAC348, and their derivatives) were provided by Y. Oshima and N. Ogawa, (Ogawa and Oshima 1990). Plasmid pM16-2, previously described as pM16-3 (Thomas, Barbey and Surdin-Kerjan 1990), was obtained from Y. Surdin-Kerjan, plasmid pLG669Z from L. Guarente (Guarente and Ptashne 1981) and plasmids pRS425CBFAP4 and pRS425CBFAP4R from C. Dang (Dang *et al.* 1992). Plasmid pKO17 contained the 3.0 kbp *Nhe*I-*Pvu*II fragment from plasmid pKO11 inserted between the *Xba*I and *Sma*I sites of YEp352 (Hill et al. 1986b). Plasmid pRB101, containing a *cep1::TRP1* disruption allele, was constructed by replacing 620 bp of *CEP1* coding region [between *Ssp*I sites at nucleotides 409 and 1029 (Baker and Masison 1990) with the *Eco*RI/*Bgl*II restriction fragment of the yeast *TRP1* gene using *Xho*I linkers. The *pho84::URA3*

disruption allele contained in plasmid pKO33 was constructed as follows. A segment of the *PHO84* gene extending from the *Hin*dIII site at position 644 to the *Xba*I site at position 1674 (Bun-Ya *et al.* 1991) was obtained by amplification of yeast genomic DNA using the polymerase chain reaction and inserted into the polylinker of pUC18. The segment extending from the *Hpa*I site (position 867) to the *Bgl*II site (position 1465) was then removed and replaced with the yeast *URA3* gene. Plasmid pDR1-7, used to prepare reference probe for the DNA-binding assays, contains a single copy of the CDEI site from the centromere of chromosome III (*CEN3*) cloned into the *Bam*HI site of pUC18 (Masison, O'Connell and Baker 1993). All *CYC1-lacZ* reporter plasmids were derived from plasmid pLG669Z. Plasmids pRB137-2, and pRB137-4 contain, in opposite orientations, the 305-bp *Rsa*I fragment spanning positions 411 to 715 of the *MET16* promoter region (Thomas, Barbey and Surdin-Kerjan 1990) inserted into the Klenow polymerase-blunted *Xho*I site of pLG669Z, the *Xho*I digestion having removed the *CYC1* UAS. Plasmid pRB137-1 lacks an insert altogether, and served as a UAS-less control. To construct reporter plasmid pKM11-1, the 305-bp *Rsa*I fragment was first cloned into the *Sma*I site of pGEM4 creating pRB136-1 which contained two tandem copies of the insert. This plasmid was digested with *Rsa*I and *Sal*I to excise a single copy of the element which was isolated and ligated between the *Sma*I and *Xho*I sites of pLG669Z, creating a reporter containing the *MET16* UAS in place of the *CYC1* UAS element and additional upstream sequences.

Site-directed mutagenesis was performed using the Altered Sites in vitro Mutagenesis System (Promega Corporation) according to the manufacture's instructions. The initial template for the mutagenesis reactions,

pKM10-3, was constructed by inserting the *Eco*R1-*Hin*dIII fragment of pRB136-1 between the *Eco*RI and *Hin*dIII sites of the phagemid pALTER-1. The template therefore contained two tandem copies of the target sequence. Conversion of the CDEI site (identical to a *Pml*I restriction site) to an *Spe*I site was achieved using the mutagenic oligonucleotide M16-47 (5'-TCATCATTT-CACTAGTGGCTAGTAAAAGAA-3'). A derivative, pKM14-1, contained the precise changes in both inserts, and was used to construct a template for the generation of additional mutants. Plasmid pKM14-1 (Amp^r) was cut with *Spe*I and religated leaving a single insert which was then removed as an *Eco*RI-*Hin*dIII fragment and ligated between the *Eco*RI and *Hin*dIII sites of pALTER-1 (Amp^S) creating pKM20-1. Using this plasmid as template, mutagenesis was performed individually with the mutagenic oligonuc-leotides M16-22-27 (5'-CATCATTTCACGNNNCTAGTAAAAGAA-3'), M16-33 (5'-TTTTTATTTTTATCACTAGTCACGTGGCTA-3'), M16-39 (5'-TCATCATTT-CACATGGCTAGTAAAAGAA-3'), and M16-48 (5'-TCATCATTTCAGCTGG-CTAGTAAAAGAA-3'). Reporter plasmids carrying these mutated elements were constructed by removing each insert as an *Rsa*I-*Sal*I fragment from the mutagenesis vector and cloning it between the *Sma*I and *Xho*I sites of pLG669Z. The precise sequence changes were confirmed by sequencing the inserts contained in the cognate reporter plasmids.

The plasmid used to construct the replacement vectors (pKM46) contained *MET16* sequences from position 1 to the *Rsa*I site at position 412 joined by a *Sma*I-*Bam*HI linker (CCCGGGATCC) to sequences from position 708 (*Acc*I site) to position 964 (*Cla*I) and cloned between the *Xba*I and *Cla*I sites of the *URA3* integrating plasmid pRS306 (Sikorski and Hieter 1989). Mutated

MET16 promoter fragments were excised from the corresponding pALTER-1 phagemids as *Rsa*I/*Bam*HI fragments and inserted at the SmaI/BamHI linker of pKM46 creating the replacement vectors, pKM47 (*met16-47*) and pKM48 (*met16-48*).

Gene disruptions and replacements. All of the *cep1* strains used in this study contained one of four disrupted *cep1* alleles. The structures and procedures used to generate the *cep1::URA3-10*, *cep1::URA3-11*, and *cep1::ura3* alleles have been described (Baker and Masison 1990; Masison and Baker 1992). The endogenous *CEP1* gene in strains YPH98, NBW7, and NBD82-1 was replaced with the *cep1::TRP1* allele in pRB101 by homologous recombination (Rothstein 1991) to create strains R31-3B, K43-T1, and K22-T8 respectively. Successful *CEP1* gene disruptions were confirmed using either Southern blotting or the polymerase chain reaction.

Disruption of the wild-type *PHO84* gene in strain K45 was achieved by excising the *pho84::URA3* allele from the pUC18 polylinker of pKO33 with *Eco*RI and *Hin*dIII and transforming the diploid yeast strain K23 to uracil prototrophy with the restricted plasmid. Tetrad analysis of one of the Ura^+ transformants (strain K45-T1) indicated that one of the two *PHO84* loci had been replaced by the disrupted version, and Ura^+ segregants were Pho^-, *i.e.*, they fail to grow on P_i-depleted medium.

Replacement of the wild-type *MET16* gene in strains K55-M47 and K56-M48 was achieved using the pop-in/pop-out gene replacement method (Rothstein 1991). For the pop-in, the integrating plasmids pKM47 and pKM48 were cut with *Bam*HI and used to transform strain K23-9A to uracil prototrophy. Transformants which had correctly integrated the plasmids were identi-

fied by Southern blotting. For each mutation, one integrant was chosen, and derivatives which had excised the plasmid (pop-outs) were selected using 5-fluoroorotic acid (Boeke, Lacroute and Fink 1984). Retention of the mutations in the genome was confirmed by Southern blotting (both mutations create new restriction sites in the *MET16* 5'-flanking DNA). [Sequences surrounding the *Acc*I site at position 708, destroyed by insertion of the *Sma*I-*Bam*HI linker in the replacement vectors, is restored during the pop-in recombination event.]

Isolation of plasmid suppressors. Plasmid suppressors were isolated in two separate screens of the YEp24-based (*URA3*) yeast genomic library of Carlson and Botstein (1982). In the first screen, diploid R33R63 was transformed to uracil prototrophy and Met$^+$ colonies were identified by replica plating onto media lacking both uracil and methionine. Plasmid dependence of the Met$^+$ phenotype was tested by plating the transformants on medium containing 5-fluoro-orotic acid to select for cells having lost the plasmid (Boeke, Lacroute and Fink 1984) and then redetermining the Met phenotype. Three of 8200 transformants had acquired a Met$^+$ phenotype that was plasmid-dependent. The three plasmids were rescued into *E. coli*. Restriction enzyme analysis revealed that two of the three plasmids were identical and all contained *CEP1*.

In the second screen, transformants of the haploid strain R31-3BR were selected directly on medium lacking both uracil and methionine. An aliquot of the transformation mix was plated on uracil single drop-out medium to estimate transformation frequency. Of a predicted 11,300 transformants, four grew on double selection medium. When tested for plasmid-dependence, the

Met$^+$ and Ura$^+$ phenotypes cosegregated in all four cases. Restriction analysis of the rescued plasmids revealed that two of the plasmids (pMAC2-1, pMAC2-2) were identical and contained an insert which overlapped that of the third plasmid (pMAC3-2). The fourth plasmid (pMAC1-3) was unique. None of the plasmids contained *CEP1*. Upon retransformation, pMAC1-3 was not able to reproducibly confer the Met$^+$ phenotype, and it was not analyzed further.

Isolation of pseudorevertants. Pseudorevertants were isolated in two different strain backgrounds, D1-1C (*cep1::URA3*) and R31-5C (*cep1::TRP1*). In the first screen, cells from 12 independent stocks of D1-1C were spread onto methionine dropout plates. Met$^+$ colonies arose after 3-4 days at a frequency of 1-4 x 10^{-5}. Eighteen isolates (12 independent) were chosen for analysis. Each pseudorevertant was backcrossed to test for dominance, and the resulting diploids were sporulated to recover all 18 suppressors in a background of the opposite mating type. A complete complementation matrix was obtained. All 18 suppressors were recessive, and 6 of them defined a single complementation group that we designated *sma1* (suppressor of *cep1* methionine auxotrophy). The remaining suppressors were weak and appeared to be under mating type control, because suppression was only observed in a haploid genetic background. That is, *MAT***a** *smaX* and *MATα* *smaX* strains were Met$^+$, but *MAT***a**/*MATα* *smaX*/*smaX* diploids were Met$^-$.

In a second screen, cells from a single culture of R31-5C were plated on methionine dropout plates, and Met$^+$ colonies arose at a frequency of 4 x 10^{-5}. Twenty-three isolates (not necessarily independent) were analyzed as before. Thirteen of the 23 suppressors were recessive, and 8 of them failed to complement *sma1*. The non-*sma1* suppressors were weak and they were not

characterized further. Pseudorevertants were customarily maintained as patches on methionine dropout plates; when spread at low cell density (*e.g.*, streaking for single colonies) plating efficiency was poor.

Acid phosphatase assays. Acid phosphatase activity was quantitated using whole cells as the enzyme source. Strains were pregrown to near saturation in media containing 3/5 the normal amount of phosphate. Cells were then diluted at least 100-fold into either high or low phosphate medium and grown to an OD_{650} of between 0.5 and 4.5. Phosphatase activity was assayed using *p*-nitrophenylphosphate as substrate. The reaction mixture contained 0.1 M sodium acetate (pH 4.2), 4.5 mg/ml *p*-nitrophenylphosphate (Sigma), and up to 0.1 ml culture in a total reaction volume of 0.50 ml. After incubation for 10 min at 37°C, reactions were terminated by the addition of 0.72 ml saturated sodium carbonate and the cells were removed by centrifugation. The amount of *p*-nitrophenol produced was determined by measuring absorbance at 420 nm. One unit of activity is defined as 1 µmole of *p*-nitrophenol liberated per min.

Northern blotting. Procedures for isolating total cellular RNA (Herrick, Parker and Jacobson 1990) and blot hybridization (Amasino 1986) were as described. For the nutritional shift experiments, cells were pregrown in synthetic complete medium supplemented with 1 mM methionine ($SCM_{1.0}$ media) then harvested by centrifugation, washed in the same medium lacking methionine (SCM_0 media), and resuspended in a small volume of SCM_0. This cell suspension was used to inoculate equal volumes of SCM_0 and $SCM_{1.0}$ to give initial culture densities of 0.5-1.0 x 10^7 cells/ml. Samples were collected periodically following the nutritional shift and used

to isolate total RNA. To analyze *GCN4*-dependent expression, cells were pre-grown for several hours in synthetic complete medium lacking all aromatic amino acids before being starved for tryptophan by adjusting the cultures to 1 mM 5-methyltryptophan (5-MT). Growth was allowed to continue and samples were removed periodically and used to isolate total RNA.

Probes used to detect various messages were labeled to high specific activity with [α-^{32}P]-dCTP using the Random Primed DNA Labeling Kit (Boehringer Mannheim) according to the manufacturer's instructions. Templates for the labeling reaction included the following gel purified fragments: for *MET16*, the 563-bp *Cla*I-*Eco*RI fragment (Thomas, Barbey and Surdin-Kerjan 1990); for *MET25* the 271-bp *Eco*RI/*Xba*I fragment (Kerjan, Cherest and Surdin-Kerjan 1986); for *ACT1* the 300-bp *Bgl*II-*Dra*I (Ng and Abelson 1980); for *HIS4* the *Sac*I-*Sph*I fragment (Donahue, Farabaugh and Fink 1982). All blots were quantitated on a Betagen Betascope 603 Blot Analyzer.

DNA-binding assays. The relative apparent binding constants for CP1 binding to the mutated CDEI sites were measured using a competition gel electrophoretic mobility shift binding assay (Baker, Fitzgerald-Hayes and O'Brien 1989). "Test" probes were synthesized by polymerase chain reaction using as template pALTER-1 plasmids containing wild-type and mutated *MET16* promoter segments and primers flanking the polylinker region (puceco, 5'-GAATTCGAGCTCGGTACCC-3'; puchind, 5'-AAGCTTGCATG-CCTGCAGGTC-3'). A "reference" DNA probe was synthesized by polymerase chain reaction using the same primer set and pDR1-7 as template. Plasmid pDR1-7 contains the CDEI site of *CEN3* cloned into the *Bam*HI site of pUC18

(Masison, O'Connell and Baker 1993). The puceco primer was end-labeled with [γ-^{32}P] ATP using polynucleotide kinase to a predicted specific activity of ~1000 Ci/mmol, and the same preparation of primer was used in each amplification reaction to generate probes of equal specific radioactivity. The resulting test and reference probes, 362 and 82 bp in length, respectively, were purified by polyacrlyamide gel electrophoresis and isolated by electroelution. DNA-binding reactions were carried out exactly as described (Masison, O'Connell and Baker 1993) using purified yeast CP1 (Baker, Fitzgerald-Hayes and O'Brien 1989). Test and reference probes were added to the reactions in various ratios. After electrophoresis, the gels were dried and all four species of DNA (bound and free test DNA, bound and free reference DNA) were quantitated simultaneously using the Betagen Betascope 603. Relative apparent equilibrium constants were calculated from the following equation (Baker, Fitzgerald-Hayes and O'Brien 1989):

$$K_{rel} = \frac{K_{test}}{K_{ref}} = \frac{T_b}{R_b} \cdot \frac{R_f}{T_f}$$

where K_{rel} is the relative apparent equilibrium constant, K_{test} and K_{ref} are the individual equilibrium constants for CP1 binding to test and reference probes, respectively, T_b and R_b are the cpm of CP1-bound test and reference probes, respectively, and T_f and R_f are the cpm of unbound (free) test and reference probes, respectively. The measured K_{rel} for the wild-type *MET16* CDEI site vs. *CEN3* CDEI was 0.65 ± 0.08 (mean ± s.e.) for 12 separate determinations. The average variation from the mean in independent determinations of K_{rel} values for the four mutant CDEI's was 40%. This approach to measuring relative binding constants is general, and requires only that test and refer-

ence DNA's differ sufficiently in electrophoretic mobility such that both bound and free forms of each are resolvable and that both probes can be synthesized using at least one common polymerase chain reaction primer.

Analysis of chromatin structure. The chromatin structure of the *MET16* promoter was analyzed essentially as described by Hull *et al.* (1991). Strains were grown in $SCM_{1.0}$ or SCM_0 medium to mid log phase, collected by centrifugation, and suspended in 15 mls 40 mM EDTA. After the addition of 100 µl of 2-mercaptoethanol, the samples were left at room temperature for 5 min. The cells were again collected by centrifugation and suspended in either $SCM_{1.0}$ or SCM_0, corresponding to their growth medium, containing 1 M sorbitol. Zymolyase-100T (ICN) was added to 1.25 mg/ml and the cells incubated for 30 min at 30°C with intermittent agitation. Cell wall digestion was stopped by addition of ice-cold hypotonic buffer (100 mM NaCl, 6 mM Tris-HCl, pH 7.4, 6 mM $MgCl_2$) containing 1 mM $CaCl_2$. The spheroplasts were pelleted by centrifugation, resuspended to 100 cell units/ml (cell units = OD_{600} x ml of original culture) in hypotonic buffer containing 1 mM $CaCl_2$, 0.05 % Triton X-100, 2 ug/ml pepstatin, and 1 mM phenylmethyl sulfonyl fluoride, then transferred to prechilled 7-ml Dounce homogenizers, and broken by six or seven strokes with the loose-fitting pestle. Aliquots, dispensed to microfuge tubes on ice, were prewarmed at 30°C for 3 min before adding micrococcal nuclease (Sigma Chemical Co.) to the final concentrations specified in the legend to Figure 10. Digestion was allowed to proceed for 3 min, then terminated with an equal volume of stop solution (1 M NaCl, 50 mM Tris-Cl pH 7.9, 2% sodium dodecyl sulfate, 50 mM EDTA). Genomic DNA was isolated as described (Hull *et al.* 1991), digested to completion with *Eco*RI and

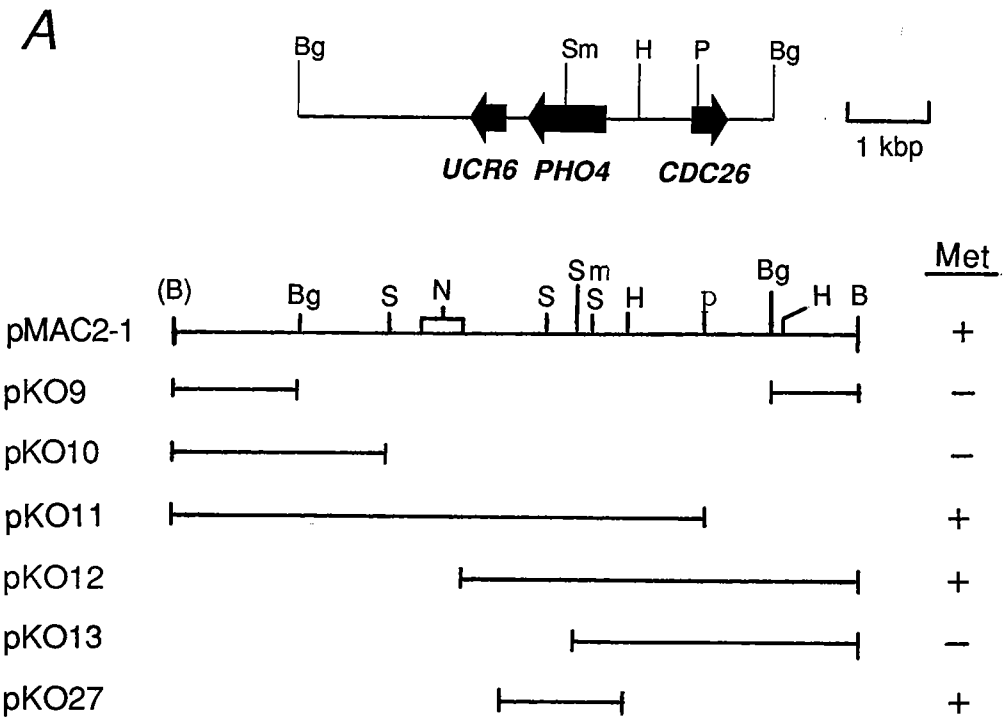
*Xba*I, and separated by agarose gel electrophoresis. The DNA was transferred to a nylon membrane and analyzed by indirect end-labeling (Wu 1980) by hybridization to the same *Eco*RI-*Cla*I *MET16* probe as used for Northern analysis. In most experiments, indirect end-labeling was also performed with a probe complementary to the region adjacent to the upstream *Xba*I site. In these cases, the pattern of nuclease cleavage sites was similar to that obtained with the *Eco*RI-adjacent probe. All samples were spiked with 10 ng lambda *Hin*dIII/*Eco*RI molecular weight markers. After probing the blot for *MET16*, the membrane was stripped and reprobed with labeled lambda DNA to detect the molecular weight standards. For each lane, a standard curve was generated and used to determine the size of the *MET16* fragments visualized in the same lane. Standard deviation of the deduced micrococcal cleavage sites, within and between experiments, ranged from 6 to 31 bp.

CHAPTER III

ISOLATION AND ANALYSIS OF SUPPRESSORS OF THE *cep1* METHIONINE AUXOTROPY

A multicopy suppressor of *cep1* methionine auxotrophy. In an attempt to identify the gene or genes limiting the growth of *cep1* disruption strains on medium lacking methionine, I screened for yeast plasmids which would suppress *cep1* methionine auxotrophy when present at high copy. In two screens of a YEp24-based gene bank (Carlson and Botstein 1982), four different plasmids were obtained which conferred methionine prototrophy (see Materials and Methods). Restriction analysis revealed that two of the suppressing plasmids contained *CEP1*. The other two plasmids (pMAC2-1 and pMAC3-2) contained overlapping inserts apparently unrelated to *CEP1*. A hybridization probe prepared from the insert of pMAC2-1 was used to probe a blot of electrophoretically separated yeast chromosomes. The probe hybridized to chromosome VI (not shown). A subsequent survey of cloned chromosome VI genes revealed a striking similarity between the restriction map of the 8.6-kbp pMAC2-1 insert and that of the *CDC26-PHO4-UCR6* locus (Figure 1A). Several pMAC2-1 subclones were tested, and the Met^+ phenotype was found to correlate with the presence of *PHO4* (Figure 1A). Furthermore, a subclone containing only the putative *PHO4* sequences carried on a single copy centromere-containing vector (pKO27) was sufficient to rescue methionine prototrophy (Figure 1C). To verify that pKO27 carried *PHO4*, it's ability to complement a *pho4* mutation was tested. Strain NBD4-1 (*pho4::HIS3*) was transformed with plasmids pKO27, a multicopy *CEP1* plasmid (pMAC3-1), and a vector control (pRIP1). Only pKO27 rescued the ability to grow on medium depleted of inorganic phosphate (Figure 1B). The multicopy *CEP1* plasmid did not suppress the P_i requirement of the *pho4* mutant even though expression of *CEP1* from a multicopy plasmid is known to result

FIGURE 1.—Analysis of plasmid suppressor pMAC2-1. (A) Restriction maps of the pMAC2-1 insert and the *PHO4* locus (Ogawa and Oshima 1990). Subclones tested for suppressor function are diagrammed below the pMAC2-1 map. The lines represent the DNA present in each construct. Plasmids pKO9, pKO10, pKO11, pKO12, and pKO13 were obtained by digesting pMAC2-1 with *Bgl*II, *Sph*I, *Pvu*II, *Nhe*I, and *Sma*I respectively, and religating. Plasmid pKO27 contains the 1.5 kbp *Acc*I-*Hin*dIII fragment (*Acc*I end filled in by Klenow polymerase) inserted between the *Hin*dIII and *Sma*I sites of pRIP1 (Parker and Jacobson 1990). Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; N, *Nhe*I; P, *Pvu*II; S, *Sph*I; and Sm, *Sma*I. (B) Complementation of a *pho4* mutation. The host strain NBD4-1 (*pho4::HIS3*) was transformed with the CEN plasmids pKO27 and pRIP1 (vector control), and the episomal plasmid pMAC3-1 which carries *CEP1*. Transformants were tested for growth on YEPD medium depleted of inorganic phosphate (YEPD-P_i). (C) Growth of *cep1* strains in media lacking methionine at high and low P_i concentrations. Host strain R31-3BR (*cep1*) was transformed with either pKO27 or pKO9 (control), and transformants tested on methionine-free plates containing the indicated amounts of P_i. R31-1A is an isogenic wild-type strain. The plates were photographed after 3 d growth at 30°C.


A
Bg
Sm
H
P
Bg
1 kbp
UCR6
PHO4
CDC26
Met
(B)
Bg
S
N
S
Sm
S
H
p
Bg
H
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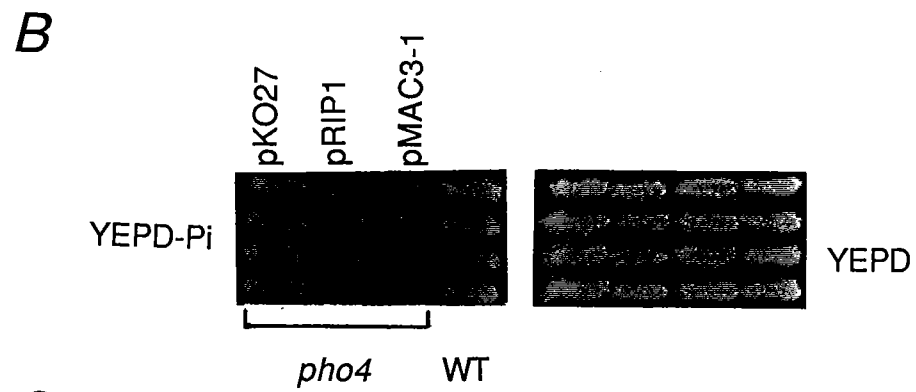
+
pKO12
+
pKO13
–
pKO27
+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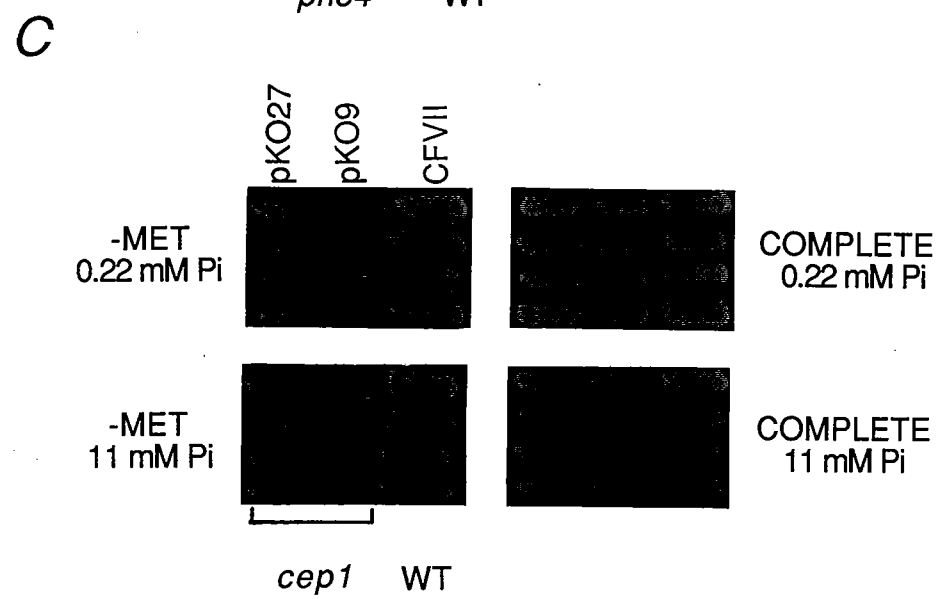
pRIP1
pMAC3-1
YEPD-Pi
YEPD
pho4
WT


C
pKO27
pKO9
CFVII
-MET
0.22 mM Pi
COMPLETE
0.22 mM Pi
-MET
11 mM Pi
COMPLETE
11 mM Pi
cep1
WT

in overproduction of CP1 (D. Masison and R. Baker, unpublished observations). Thus, extrachromosomal copies of *PHO4* are sufficient to suppress the methionine auxotrophy of a *cep1* mutant, but multiple copies of *CEP1* are unable to suppress the Pho$^-$ phenotype of a *pho4* mutant.

The Met prototrophic phenotype correlates with derepression of the *PHO* regulon. *PHO4* is part of a regulatory network (Figure 2) consisting of several genes (Toh-e 1989; Vogel and Hinnen 1990). *PHO80* is a negative regulatory element whose gene product is thought to function by sequestering PHO4 protein in an inactive form when P_i is not limiting. When P_i becomes limiting, the repressive effect of PHO80 is relieved and PHO4 becomes free to activate transcription of several target genes. The response to P_i limitation requires the product of *PHO81*, which appears to be a sensor of intracellular P_i levels. Among the genes activated by PHO4 are *PHO5* and *PHO84*. *PHO5* encodes a repressible acid phosphatase (rAPase) and *PHO84* a low K_m phosphate permease (Bun-Ya *et al.* 1991). The expression of both *PHO5* and *PHO84* requires a second factor encoded by *PHO2* (also known as *BAS2* or *GRF10*) (Arndt, Styles and Fink 1987; Tamai, Toh-e and Oshima 1985; Yoshida, Ogawa and Oshima 1989).

To determine if *PHO5* was derepressed in *cep1* strains carrying extrachromosomal copies of *PHO4*, rAPase levels were measured in transformants of a *cep1* strain carrying single or multicopy *PHO4* plasmids and grown in high (repressing) P_i medium (Table 2). The host strain used for these experiments also carried a *pho3* mutation, eliminating interference from the constitutive acid phosphatase encoded by this gene. Transformants carrying the CEN plasmid pKO27 expressed normal repressed levels of rAPase, but cells

FIGURE 2.—The *PHO* Regulon. Shown is the diagram of the regulatory circuit governing expression of genes required by yeast for growth under limiting P_i concentrations [Originally published by Vogel and Hinnen (1990)]. Several structural genes are shown and include *PHO5* encoding an acid phosphatase, *PHO8* encoding an alkaline phosphatase, and *PHO84* encoding the high-affinity P_i transporter. The structural genes are regulated at the level of transcription by two positive factors encoded by *PHO4* and *PHO2*. In the presence of sufficient P_i, the activity of PHO4 is inhibited by the action of two negative factors encoded by *PHO80* and *PHO85*. In the absence of sufficient P_i, the product of the *PHO81* gene is required to transmit a signal which removes negative regulation of PHO4 by the PHO80/PHO85 system. Also indicated are possible autoregulation of *PHO2* expression and positive regulation of *PHO81* expression by PHO4 and PHO2.

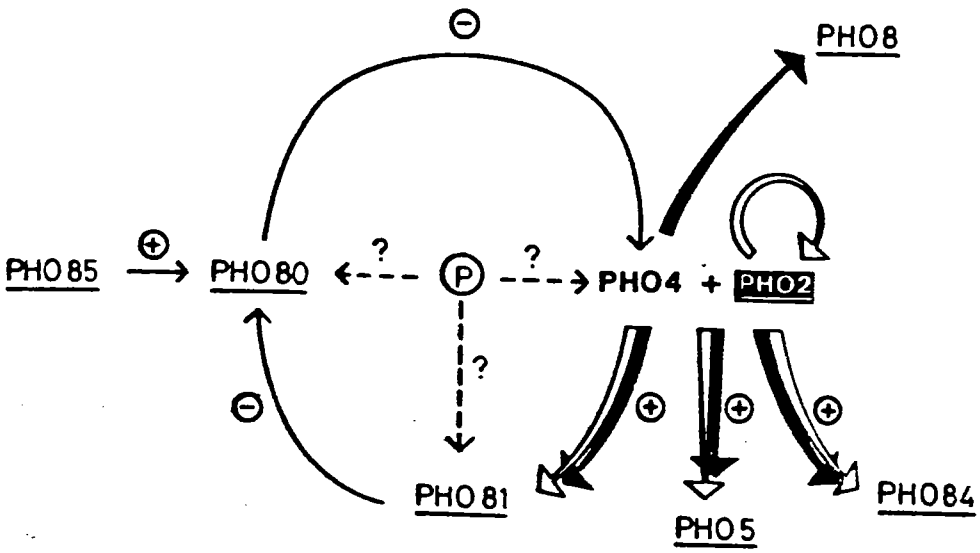

PHO8
PHO85
PHO80
P
PHO4
PHO2
PHO81
PHO5
PHO84

TABLE 2

rAPase activity of *PHO4* plasmid-bearing strains

Strain	Genotype [plasmid]	Growth Conditions	rAPase Activity[a]
NBW7	*pho3*	Hi P_i	0.92 ± 0.09 (3)
NBW7	*pho3*	Lo P_i	84.1 ± 2.8 (3)
K43-T1	*pho3 cep1*	Hi P_i	0.60 ± 0.05 (4)
K43-T1	*pho3 cep1*	Lo P_i	39.7 ± 4.2 (4)
K43-T1	*pho3 cep1* [pRIP1]	Hi P_i	1.13 ± 0.89 (4)
K43-T1	*pho3 cep1* [pKO27]	Hi P_i	0.60 ± 0.15 (4)
K43-T1	*pho3 cep1* [YEp352]	Hi P_i	0.34 ± 0.08 (4)
K43-T1	*pho3 cep1* [pKO17]	Hi P_i	5.48 ± 1.2 (4)

[a] mU/OD_{660} cells; mean ± SD (no. determinations)

carrying the multicopy *PHO4* plasmid pKO17 expressed significantly elevated levels of rAPase. Therefore, overexpression of *PHO4* in these strains leads to derepression of *PHO5* and presumably other genes activated by PHO4. Also, while overexpression of *PHO4* suppresses *cep1* methionine auxotrophy, the elevated level of PHO4 apparently is insufficient to derepress *PHO5* to the level achievable through P_i limitation.

The rAPase derepression in cells overexpressing *PHO4* probably occurs due to an imbalance between the level of PHO4 and that of its negative regulator PHO80 (Yoshida, Ogawa and Oshima 1989). Since mutations in either *PHO80* or *PHO4* can also cause inappropriate *PHO* gene derepression (Ogawa and Oshima 1990), we tested two such mutations to determine if they would also suppress *cep1* methionine auxotrophy. Strain NBD82-1 carries the *PHO4^c-1* allele. Repressible APase activity in this strain is derepressed in high phosphate medium by approximately 10-fold compared to the wild-type strain NBW7 (Table 3). A *PHO4^c-1 cep1::TRP1* double mutant was obtained by disrupting *CEP1* in NBD82-1. This strain (K22-T8) was phenotypically Met$^+$, although not to the full extent of a wild-type strain (Figure 3B), and it expressed rAPase constitutively (Table 3). The level of rAPase expression in the *cep1 PHO4^c* double mutant was only about half that of the *CEP1 PHO4^c* strain NBD82-1. When K22-T8 was backcrossed to a *cep1::URA3* strain, Met$^+$ and Pho^c phenotypes cosegregated (not shown). To prove that suppression was genetically linked to *PHO4^c-1*, K22-T8 (*PHO4^c-1 cep1::TRP1*) was mated with a *pho4::HIS3 cep1::URA3* strain and segregation analysis performed. Fifty of 51 His$^-$ segregants were Met$^+$, while 50 of 50 His$^+$ segregants were Met$^-$, demonstrating tight linkage between suppression and *PHO4^c*. Next I tested whether

TABLE 3

rAPase activities

Strain	Genotype[a]	rAPase Activity[b]
NBW7	*pho3*	0.92 ± 0.09(3)
NBD82-1	*pho3 PHO4*^{c}	10.7 ± 1.2(3)
K22-T8	*pho3 PHO4*^{c} *cep1*	5.85 ± 1.3(5)
K23-4A	wild-type	4.84 ± 0.54 (4)
K23-4B	*pho80*	171 ± 16 (6)
K23-4C	*cep1*	6.47 ± 0.37 (4)
K23-4D	*cep1 pho80*	88.6 ± 11 (6)
D1-6C	wild-type	4.82 ± 2.0 (3)
K6-25B	*cep1*	5.38 ± 1.1 (3)
K6-25A	*cep1 sma1*	53.1 ± 5.7 (3)
K45T1-4B	*pho84*	73.9 ± 8.1 (4)
K45T2-1D	*cep1 pho84*	39.6 ± 4.5 (4)

Growth conditions: high P_i

[a] Complete genotypes given in Table 1.

[b] mU/OD_{660} cells; mean ± SD (no. determinations)

loss of the negative regulator PHO80 would result in suppression. Diploid K23 is heterozygous for null alleles of both *cep1* and *pho80*. Tetrad analysis of K23 revealed that the Met^+ phenotype segregated predominantly $3^+:1^-$ (Table 4), as would be expected if the *pho80::LEU2* allele suppressed the methionine auxotrophy of the *cep1::TRP1* segregants, *i.e.*, all of the *CEP1* and half of the *cep1::TRP1* segregants were Met^+. All of the *cep1::TRP1 pho80::LEU2* segregants (*i.e.*, Trp^+ Leu^+) were Met^+ (30/30). The suppressed phenotype of a typical *pho80::LEU2 cep1::TRP1* segregant is shown in Figure 3B. The phosphatase activities of spores obtained from a tetratype K23 tetrad are given in Table 3. Again, derepression of rAPase correlates with suppression of *cep1* methionine auxotrophy (K23-4D), and the level of rAPase in the *cep1* background is approximately half that of the wild-type segregant (compare strains K23-4D and K23-4B). The higher background activity of acid phosphatase in these strains (about 5 mU/OD_{660} cells) was due to the presence of an active *PHO3* allele.

The prototrophy of *cep1 PHO4^c* and *cep1 pho80* double mutants indicated that a single chromosomal copy of *PHO4* was sufficient to suppress *cep1* methionine auxotrophy when negative regulation by *pho80* was abrogated. Next, I asked if the auxotrophy could be suppressed under normal physiological conditions, *i.e.* , by limiting phosphate. A master plate containing wild-type and *cep1* strains was replicated onto a series of plates containing various concentrations of methionine and P_i. As shown in Figure 1C, the wild-type strain and a *cep1* strain carrying *PHO4* on a CEN plasmid (pKO27) were able to grow in the absence of added methionine at both high and low P_i concentrations, but neither of these P_i concentrations allowed growth of a *cep1* strain

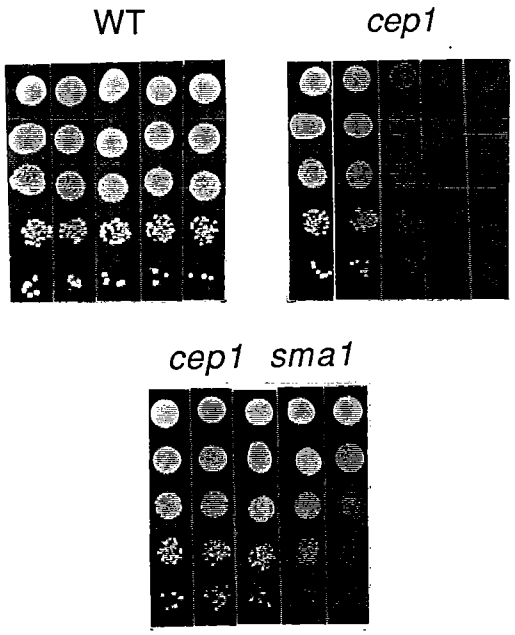
carrying only the control plasmid (pKO9). Colony staining (Toh-e and Oshima 1974) demonstrated that rAPase was indeed derepressed in all strains on the low P_i plate. Therefore while the low P_i concentration was sufficient to derepress *PHO5*, this limiting P_i concentration was insufficient to achieve suppression of *cep1* methionine auxotrophy by PHO4.

Spontaneous suppressors of *cep1* methionine auxotrophy. In working with *cep1* deletion mutants, it was observed that patches of cells replicated onto methionine-free plates frequently gave rise to Met^+ papillae. Since true reversion would be impossible, these pseudorevertants were presumed to contain extragenic suppressor mutations. To analyze the phenomenon in more detail, a number of independent pseudorevertants, isolated in two different *cep1* genetic backgrounds, were characterized (see Materials and Methods). The pseudorevertants arose at a frequency of 1-4 x 10^{-5}, and approximately one third of them defined a single recessive complementation group we named *sma1* (suppressor of *cep1* methionine auxotrophy). Meiotic mapping revealed that *sma1* was tightly centromere-linked, displaying a second division segregation frequency of only 0.7% (ditype:tetratype, 157:2) when scored against *trp1* . [The second division segregation of *trp1* is itself 0.9% (Mortimer and Hawthorne 1969).] Suppression by *sma1* was quantitatively similar to that observed with $PHO4^c$ and *pho80::LEU2* (Figure 3). The non-*sma1* suppressor mutations conferred a very weak Met^+ phenotype which made analysis difficult. These suppressors were not studied further.

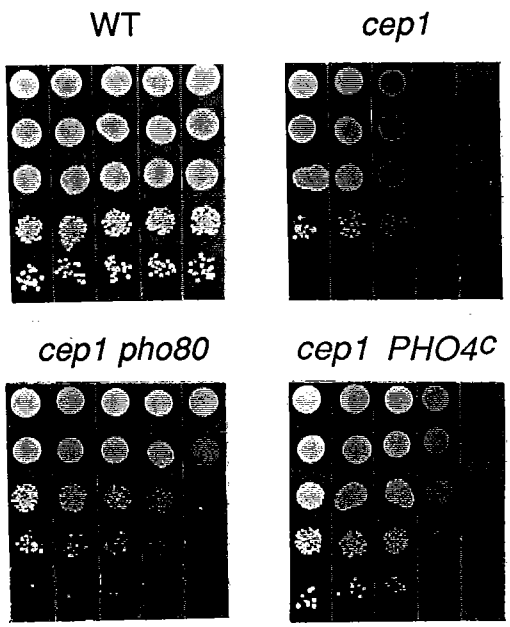
Acid phosphatase assays revealed that *sma1* strains had a Pho^c phenotype; rAPase levels were derepressed about 10-fold under high-P_i growth conditions (Table 3, K6-25B vs. K6-25A). This suggested that *sma1* might act

FIGURE 3.—Met phenotypes of suppressor strains. After growth in complete synthetic media to near saturation, cells were pelleted and resuspended in water to a density of 2×10^7 cells/ml. Ten-fold serial dilutions of each were prepared and 10-μl aliquots of the undiluted and diluted samples were spotted on plates containing various concentrations of methionine. Plates were photographed after 3 d growth at 30°C. Each panel is a composite photograph for each strain. From left to right in each panel, medium containing 300, 30, 3, 0.3, and 0 μM methionine. Normal synthetic medium contains 270 μM methionine. (A) D1-6C (wild-type), D1-1C (*cep1::URA3*), F13α (*cep1::URA3 sma1*). (B). R31-1A (wild-type), R31-3BR (*cep1::TRP1*), K23-4D (*cep1::TRP1 pho80::LEU2*), K22-T8 (*cep1::TRP1 PHO4^c-1*).

A.



B.



through PHO4. Genetic tests confirmed that suppression by *sma1* was *PHO4*-dependent; *cep1 sma1 pho4* triple mutants were Met$^-$ (not shown). The recessive Pho^c phenotype, *PHO4*-dependence, and tight centromere linkage led me to suspect that *sma1* mutations might be alleles of *pho80*. To test this, a *cep1 sma1* strain was crossed to a *cep1 pho80::LEU2* strain (diploid K47). The Pho^c phenotype segregated 4$^+$:0$^-$ in 19 of 19 tetrads and 75 of 76 segregants were Met$^+$ (Table 4). Since *sma1 pho80* segregants were not obtained, I concluded that they were allelic.

Epistasis studies. The preceding experiments demonstrated a clear correlation between derepression of the *PHO* regulon (assessed by rAPase levels) and the suppression of *cep1* methionine auxotrophy. But are these two phenotypes independent, or are they causally linked? To address this question, I investigated whether or not *PHO* genes other than *PHO4* were required for the observed suppression. First I tested the regulatory genes *PHO81* and *PHO2*. Diploid K37 is homozygous for *cep1* and heterozygous for both *sma1* and *pho81::HIS3*. K37 tetrads segregated methionine prototrophy 2:2 (Table 4), and about half (16/30) of the His$^+$ spores were also Met$^+$. This result indicated that suppression by *sma1* (*pho80*) was independent of *PHO81*. *PHO2* dependence was tested in the same manner. Diploid K39 is homozygous for *cep1* and heterozygous for *pho80::LEU2* and *pho2::LEU2*. In contrast to the *pho81* heterozygote, K39 segregated methionine prototrophy mostly 1$^+$:3$^-$, and all (38/38) *pho2* spores were Met$^-$ (Table 4). [*PHO2* was scored by assaying growth on P_i-depleted medium.] This segregation pattern indicated that suppression by *pho80* required cosegregation of the wild-type *PHO2* allele. In the single tetrad where Leu$^+$ segregated 2:2 and the two Leu$^+$ spores were likely to be

TABLE 4

Segregation Analysis

Strain	Genotype[a]	Tetrad Class (Met$^+$:Met$^-$)				
		4:0	3:1	2:2	1:3	0:4
K23	*cep1/CEP1 pho80/PHO80*	5	20	3	0	0
K47	*cep1/cep1 sma1/SMA1 pho80/PHO80*	18[b]	1	0	0	0
K37	*cep1/cep1 sma1/SMA1 pho81/PHO81*	0	0	15	0	0
K39	*cep1/cep1 pho80/PHO80 pho2/PHO2*	0	0	2	16	1
K52	*cep1/cep1 pho80/PHO80 pho3pho5/PHO3PHO5*	0	0	15	0	0
K45	*cep1/CEP1 pho80/PHO80 pho84/PHO84*	23	28	1	0	0

[a] Complete genotype given in Table 1.

[b] Pho^c segregated 4:0 in 19/19 tetrads.

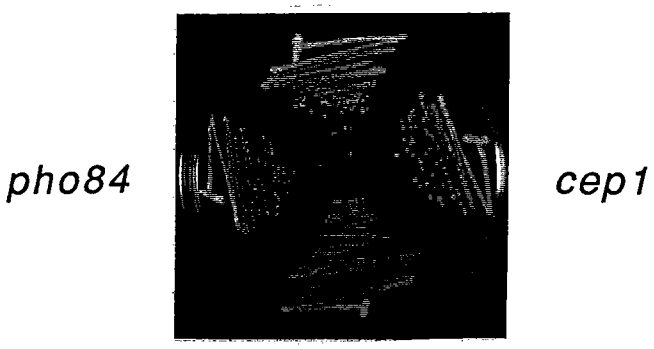
pho80::LEU2 pho2::LEU2 double mutants, both were Met$^-$. These results demonstrate that the gene product of *PHO2* but not *PHO81* is required for the *PHO4*-dependent suppression of *cep1* methionine auxotrophy.

Next tested were *PHO5* and *PHO84*, two downstream targets of *PHO4*. While it was not obvious how *PHO5* (rAPase) derepression could affect methionine biosynthesis, prototrophy correlated perfectly with high rAPase levels and *PHO5* transcription was known to be *PHO2*-dependent. Tetrad analysis of diploid K52 (*cep1/cep1 pho80::LEU2/PHO80 pho5/PHO5*) ruled out the formal possibility that suppression required *PHO5*. Methionine prototrophy segregated 2:2, and all (30/30) *pho80* (Leu$^+$) spores were Met$^+$ regardless of their allele at *pho5* (Table 4). The rationale for testing *PHO84* stemmed from the finding of Thomas, Jacquemin and Surdin-Kerjan (1992) that *cep1* mutants lacked sulfate permease activity. Since *PHO84* encodes a phosphate permease, one possible suppression mechanism would be gratuitous sulfate transport via the *PHO84* permease. Also, *PHO84* expression is *PHO2*-dependent. To test for *PHO84*-dependence of suppression, *PHO84* was disrupted in strain K23. K23 is heterozygous for both *cep1::TRP1* and *pho80::LEU2* and segregates Met$^+$ mostly 3$^+$:1$^-$ (above). The resulting disruptant (K45) was thus triply heterozygous *pho84::URA3/+*, *cep1::TRP1/+*, and *pho80::LEU2/+*. If *PHO84* were not required for suppression (by *pho80*), K45 should yield tetrads in which methionine prototrophy segregates 3$^+$:1$^-$ (as for K23), and all Leu$^+$ spores would be Met$^+$. If, on the other hand, *PHO84* were required for suppression, the frequency of Met$^+$ spores would be reduced, and the proportion of tetrads segregating Met 3$^+$:1$^-$ would be significantly decreased. Unexpectedly, the segregation pattern observed corresponded to neither of

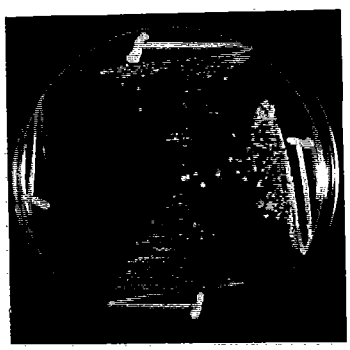
these predictions. Methionine prototrophy segregated mostly $4^+:0^-$ and $3^+:1^-$ (Table 4), suggesting that *pho84* was itself a suppressor of *cep1* methionine auxotrophy. Indeed, all (104/104) Leu^+ (*pho80*) spores were Met^+ regardless of their *PHO84* allele, and virtually all (103/104) Ura^+ (*pho84*) spores were Met^+ regardless of their *CEP1* allele. Again suppression correlated with derepression of the *PHO* regulon, as all Ura^+ segregants had elevated rAPase activities. Acid phosphatase activities of two *pho84::URA3* segregants are reported in Table 3. As before, the presence of the *cep1* allele reduced rAPase activity by about fifty percent. A second surprise was that *cep1 pho84* double mutants were Pho^+, indicating that *cep1* suppressed *pho84* for the ability to grow on P_i-depleted medium. The mutual suppression of *cep1* and *pho84* is shown in Figure 4.

Structural requirements of the *PHO4* suppressor. The facts that PHO4 and CP1 are structurally similar and that PHO4 can suppress the requirement for CP1 in methionine biosynthesis imply that both proteins normally carry out similar functions. Since the only activity PHO4 appears to possess is a transcriptional activating function, the results suggest that CP1 is also involved in activating transcription. However, CP1 and PHO4 probably activate transcription via distinct mechanisms, since PHO4 possesses a potent activation domain (Ogawa and Oshima 1990), while CP1 apparently does not (Thomas, Jacquemin and Surdin-Kerjan 1992). With respect to suppression, it is possible that the PHO4 activation domain is dispensable and that the b-HLH domain, the only region of homology shared by the two proteins, is sufficient to rescue methionine prototrophy. The HLH domain of CP1 is known to be required for CP1's role in methionine biosynthesis and chromosome segrega

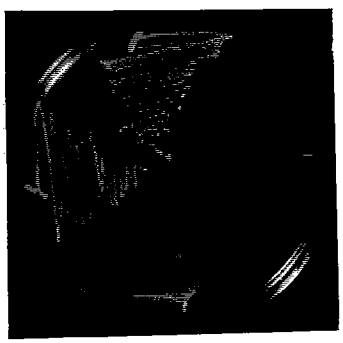
FIGURE 4.—Mutual suppression by *cep1* and *pho84*. Segregants from a tetratype K45 tetrad were streaked on synthetic media lacking methionine (-Met) and YEPD medium depleted of P_i (YEPD-P_i). Plates were incubated at 30°C for 3 d (YEPD and YEPD-P_i) or 5 d (-Met).



YEPD



YEPD-Pi



-MET

tion (Masison, O'Connell and Baker 1993), and thus it is likely that PHO4 also requires it's HLH domain to suppress the *cep1* methionine requirement. However, it is possible that PHO4 also utilizes its activation domain to restore expression to *MET* genes, thereby bypassing the normal CP1-mediated regulatory mechanism.

To determine which domains of PHO4 were required for suppression, mutant alleles of the *PHO4* gene, previously characterized by Ogawa and Oshima (1990) were examined for suppressor activity. These alleles, contained on high- and low-copy-number vectors, were tested in three strains: a wild-type strain, a *cep1* mutant, and a *cep1 pho4* double mutant. The *CEP1*-containing plasmid pDR28ARS was used as a positive control, while plasmids containing an early frame-shift allele of *PHO4* (pAC348-F16 and pAC331-F16) served as negative controls. None of the plasmids modified the methionine-independent growth phenotype of the wild-type strain, indicating the absence of dominant-negative effects on the endogenous *CEP1* gene (Table 5). Except for a few cases noted below, the methionine phenotypes of the *cep1* and *cep1 pho4* strains were affected to similar extents by each plasmid. As already demonstrated, plasmids containing the wild-type *PHO4* gene suppressed the *cep1* methionine auxotrophy, and not surprisingly, so did plasmids containing a constitutive *PHO4* allele (*Δ163-202*). In contrast, a 4-amino acid insertion into helix I of the b-HLH domain (L265), or deletion of the amino-terminal transcriptional activation domain (deletions Δ4-109, Δ4-162, and Δ4-171), abolished suppression. The results indicate that PHO4 requires both its activation and b-HLH domains to restore methionine-independent growth to *cep1* strains.

TABLE 5

Suppression of the *cep1* methionine auxotrophy by various *PHO4* alleles

		Methionine-free growth phenotype[a]		
Plasmid[b]	Description[c]	Wild-type[d]	*cep1*[d]	*cep1 pho4*[d]
pDR28ARS	*CEP1*	++	++	++
pAC331	*PHO4*	++	+	++
pAC348	*PHO4*	++	+/-	-
pAC331-F16[e]	frameshift (null)	++	-	-
pAC348-F16[e]	frameshift (null)	++	-	-
pAC331-L265	linker insertion in HLH domain	++	-	-
pAC348-L265	linker insertion in HLH domain	++	-	-
pAC331-Δ163-202	deletion of PHO80-interaction domain	++	+	+
pAC348-Δ163-202	deletion of PHO80-interaction domain	++	+/-	-
pAC331-Δ4-109	deletion of activation domain	++	-	-
pAC348-Δ4-109	deletion of activation domain	++	-	-
pAC331-Δ4-162	deletion of activation domain	++	-	-
pAC348-Δ4-162	deletion of activation domain	++	-	-

TABLE 5 cont'd

pAC331-Δ4-171	deletion of activation and PHO80-interaction domains	++	+/-	-
pAC348-Δ4-171	deletion of activation and PHO80-interaction domains	++	+	-

[a] Cells were replica plated onto synthetic complete media lacking methionine and grown for several days at 30°C. Phenotypes were scored as ++ for wild-type growth, + for intermediate growth, +/- for weak growth, and - for no growth

[b] Plasmid pDR28ARS is a centromere containing vector (Masison *et al.* 1993). The pAC348 plasmid series is maintained in single copy and the pAC331 series at multiple copies.

[c] For a complete description of the *PHO4* alleles see Ogawa and Oshima (1990).

[d] Isogenic strains used as hosts were: NBW7-1 (Wild-type), *cep1* (K43-T1), *cep1 pho4* (K54-T3)

[e] Allele *F16*, originally described as *L16* by Ogawa and Oshima (1990), was thought to carry a 4-amino acid insertion at nucleotide position 16 of the *PHO4* coding region. However, DNA sequencing and restriction analysis revealed the published mutation lacked the *Bam*HI linker, and instead carried a 4-bp insertion (data not shown).

In a few cases, plasmids which failed to confer a Met^+ phenotype upon the *cep1 pho4* mutant, were able to restore methionine-independent growth to the *cep1* mutant. These plasmids appeared to work through the endogenous PHO4 protein by titrating PHO80. Single-copy plasmids containing the wild-type allele (pAC348) or the constitutive allele (pAC348-Δ163-202) exhibited this pattern of suppression. The results obtained for the wild-type allele reinforced the extremely tight dosage dependence of suppression; loss of the chromosomal gene simultaneously reduced the copy number from two to one and eliminated suppressor activity. Surprisingly, the single-copy plasmid containing the *Δ163-202* allele behaved like the plasmid carrying the wild-type allele, suggesting that the Δ163-202 protein interacts weakly with the PHO80 protein. Also exhibiting this pattern of suppression were plasmids carrying the *Δ4-171* allele. Since this deletion removes part of the domain required to interact with PHO80, suppression by this allele would seem to be accomplished by a mechanism other than titration of PHO80 protein. The data can be reconciled as follows. In the *cep1* strain, the Δ4-171 protein (which still contains the dimerization and DNA-binding domains) is able to form a heterodimer with wild-type PHO4 protein. The heterodimer lacks one PHO80 interaction domain and thus may be poorly regulated. However, since the heterodimer still contains a normal DNA-binding domain and one activation domain, it is able to carry out its suppressor function. In the *cep1 pho4* strain, the Δ4-171 protein is unable to suppress, because it lacks a wild-type partner. Failure of the smaller deletion alleles to suppress the methionine requirement of the *cep1* strain probably reflect the presence of an intact PHO80-interaction domain. Although other interpretations are possible, the results are

most consistent with PHO4 utilizing its activation domain to bypass the requirement for CP1.

CHAPTER IV

STUDIES ON THE INVOLVEMENT OF CP1 IN THE REGULATED EXPRESSION OF THE *MET16* AND *MET25* GENES

The 5'-flanking region of the *MET16* gene confers pathway-specific regulation on a reporter gene. Disruption of the gene (*CEP1*) encoding the yeast centromere binding protein CP1 renders strains unable to grow in the absence of methionine, presumably due, in part, to the lack of two activities essential for methionine biosynthesis, sulfate transport and PAPS reductase (Thomas, Jacquemin and Surdin-Kerjan 1992). The absence of PAPS reductase activity in *cep1* strains is due to the inability to properly express *MET16*, the gene encoding this activity (Thomas, Jacquemin and Surdin-Kerjan 1992). To identify cis-acting elements important for CP1-dependent regulation, a reporter gene containing 305 bp of *MET16* 5'-flanking DNA in place of the UAS element of the *CYC1-lacZ* reporter plasmid pLG669Z (Guarente and Ptashne 1981) was constructed. The position of the single CDEI site within this fragment and the sequences immediately surrounding it are shown in Figure 5. Yeast strains carrying various reporter plasmids (Figure 5) were assayed for β-galactosidase activity following growth under conditions selective for the plasmid and either repressive (1 mM methionine) or derepressive (0.05 mM methionine) for *MET* gene expression. In the absence of its UAS element, the *CYC1-lacZ* reporter was expressed at a low level that varied neither with respect to the genetic background of the host strain nor with the methionine concentration of the medium (Table 6, plasmid pRB137-1). In contrast, when the *MET16* upstream region replaced the *CYC1* UAS (pRB137-2), β-galactosidase activity in the wild-type strain was regulated in response to methionine availability. In its normal orientation relative to the initiation site, the element directed a several hundred fold increase in activity upon methionine limitation (Table 6). Thus, this 305 bp of DNA contained all the information

FIGURE 5.—Structure of *MET16-CYC1-lacZ* reporter genes. (Top) A schematic of the *MET16* locus is shown with the coding region depicted as a solid arrow. The boxes (not to scale) represent the binding sites for CP1 (cross-hatched) and GCN4 (solid) and the numbers indicate distance, in base pairs, relative to the translation initiation codon. To construct the reporter plasmids, a 305-bp *Rsa*I fragment from the *MET16* 5'-flanking DNA was inserted between the filled-in *Xho*I sites of plasmid pLG670Z (Guarente and Ptashne 1981). (Center) *CYC1-lacZ* reporter plasmids showing the presence and orientation of the *MET16 Rsa*I fragment within each construct. Plasmid pKM11-1 is essentially identical to pBR137-2 except that it lacks all *CYC1* sequences upstream of the *Xho*I site. Deletion of this segment places the *URA3* gene immediately upstream of the *MET16* sequence. (Bottom) Site-directed mutations were made within the CDEI site of the *MET16 Rsa*I fragment and the mutated *MET16* elements inserted in place of the wild-type element in pKM11-1. The specific sequence alterations are shown with the 8-bp CDEI consensus underlined. Lowercase letters denote changes from the wild-type sequence and the hyphen indicates the additional 1-bp deletion in *met16-33*. The relative affinities of CP1 for the altered elements (K_{rel}'s) were determined by an in vitro DNA-binding assay (see Materials and Methods) and are expressed relative to the wild-type sequence.

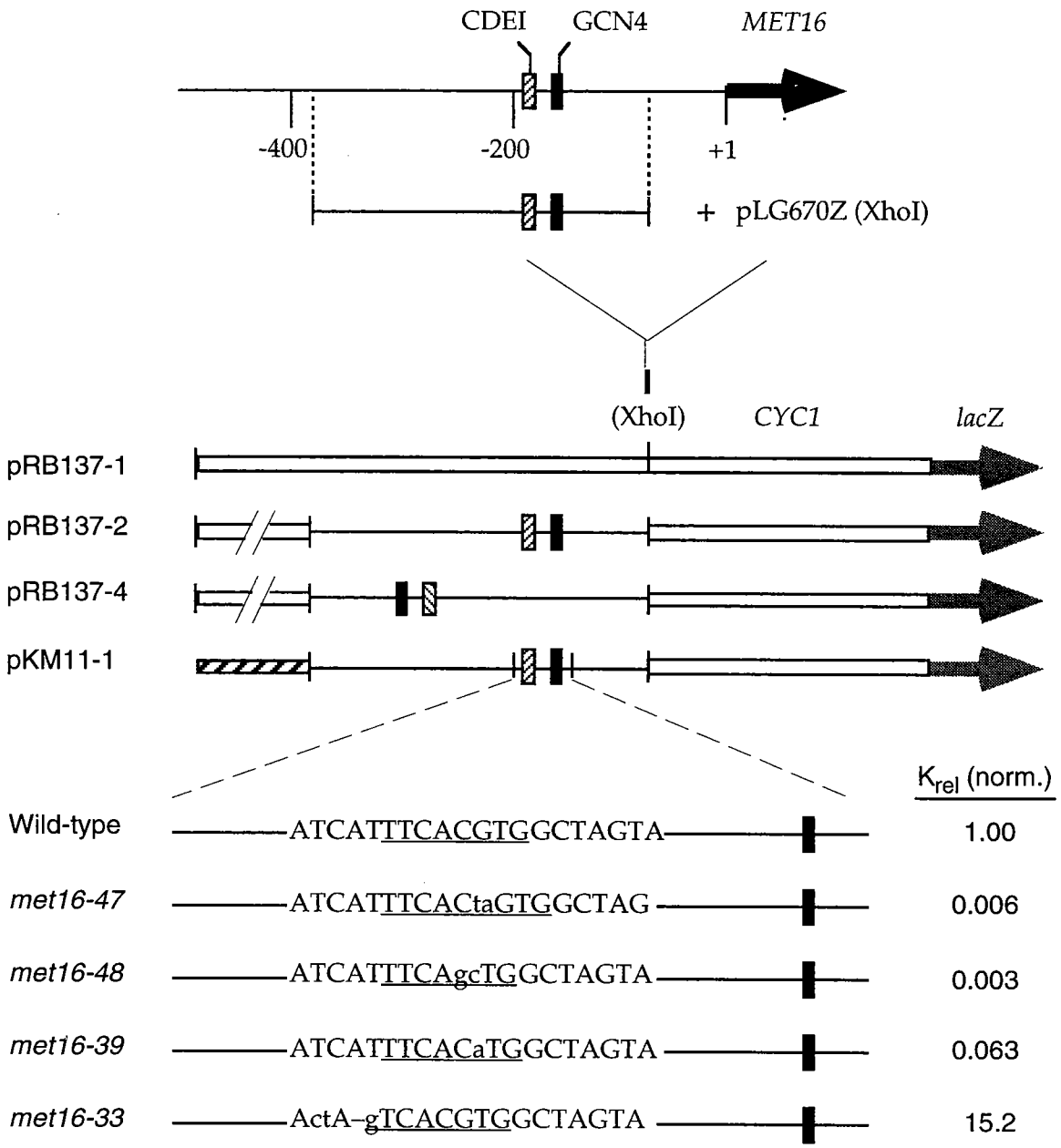

CDEI
GCN4
MET16
-400
-200
+1
+ pLG670Z (XhoI)
(XhoI)
CYC1
lacZ
pRB137-1
pRB137-2
pRB137-4
pKM11-1
Krel (norm.)
Wild-type
ATCATTTCACGTGGCTAGTA
1.00
met16-47
ATCATTTCACtaGTGGCTAG
0.006
met16-48
ATCATTTCAgcTGGCTAGTA
0.003
met16-39
ATCATTTCACaTGGCTAGTA
0.063
met16-33
ActA–gTCACGTGGCTAGTA
15.2

TABLE 6

β-galactosidase activities of *MET16-CYC1-lacZ* reporter genes

		β-galactosidase activity[a]		
Reporter	[Met][b]	Wild-type	*cep1*	*cep1 pho80*
pRB137-1	1.0 mM	0.47 ± 0.13 (4)	0.58 ± 0.15 (3)	0.40 ± 0.04 (2)
	0.05 mM	0.58 ± 0.17 (5)	0.60 ± 0.19 (3)	0.94 ± 0.19 (2)
pRB137-2	1.0 mM	0.18 ± 0.12 (5)	0.13 ± 0.02 (3)	0.22 ± 0.08 (7)
	0.05 mM	35.3 ± 8.4 (6)	0.37± 0.04 (3)	3.9 ± 3.2 (9)
pRB137-4	1.0 mM	0.43 ± 0.13 (4)	0.56 ± 0.13 (3)	0.66 ± 0.07 (6)
	0.05 mM	6.1 ± 2.4 (3)	0.57 ± 0.13 (3)	3.8 ± 2.5 (9)

[a]Cultures were grown to an O.D.$_{600} \geq 2.5$ before β-galactosidase activity was measured in whole cells (Rose, Winston and Hieter 1990). β-galactosidase activities in Miller units are reported as the mean ± standard deviation with the number of determinations in parentheses Host strains were: wild-type, K23-9A; *cep1*, K23-9B; *cep1 pho80*, K23-9D.

[b]In synthetic complete medium lacking uracil.

sufficient to confer methionine-responsive expression to the reporter gene.

As was found for the chromosomal *MET16* gene (Thomas, Jacquemin and Surdin-Kerjan 1992), expression of the *MET16* reporter construct depended on CP1. When *cep1* strains were grown under derepressing conditions, only a very low level of β-galactosidase activity was detected (Table 6). The low activity observed was not due to loss or mutation of the reporter plasmid since full activity could be restored to reporter-carrying *cep1* strains by transformation with a plasmid carrying *CEP1* (data not shown). The activity detected in the *cep1* strain was 100-fold lower than that measured in the wild-type strain, consistent with the strong CP1-dependence of *MET16* expression observed previously (Thomas, Jacquemin and Surdin-Kerjan 1992), and implying that CP1 acts at the level of transcription initiation.

The methionine auxotrophy of *cep1* strains is suppressed by mutations which cause constitutive activation of PHO4, a transactivator of genes involved phosphate metabolism (O'Connell and Baker 1992). Because CP1 and PHO4 are structurally similar and possess nearly identical DNA-binding specificity, it is presumed that PHO4 suppresses by binding to promoter CDEI sites and activating *MET* gene expression. This was tested by measuring expression of the *MET16* reporter construct in a *cep1 pho80* double mutant. PHO80 is a negative regulator of PHO4, and *pho80* mutations lead to constitutive activation of PHO4. β-galactosidase activity was partially restored in the *cep1 pho80* double mutant (Table 6). When present in the suppressor strain, the reporter expressed one tenth the level of β-galactosidase activity as observed in the wild-type strain, consistent with the observation that *pho80* only partially suppresses the methionine requirement (O'Connell and Baker 1992).

Surprisingly, the PHO4-mediated activation was observed only under conditions of methionine limitation, even though PHO4 is constitutively active in this strain. Either PHO4-mediated activation requires at least one other factor that is activated by methionine starvation, or PHO4 is unable to overcome AdoMet-mediated repression. In any event, the reporter activity observed in the *cep1 pho80* strain supports the view that PHO4-mediated suppression of *cep1* methionine auxotrophy results from restoration of CP1-dependent gene transcription.

Plasmid pRB137-4 carries the *MET16* promoter fragment in the opposite orientation (Figure 5). β-galactosidase expression driven by the inverted element was still regulated in response to methionine availability, but in this orientation the promoter fragment conferred weaker UAS activity (Table 6). In comparison to its native orientation, the inverted element was only 17% as effective in activating the reporter gene; however, activity was still completely dependent on CP1, and as before, was restored in the *cep1 pho80* suppressor strain. Interestingly, in the double mutant, reporter activity was unaffected by the orientation of the *MET16* UAS, suggesting that CP1- and PHO4-mediated regulation are mechanistically different. That is, PHO4 does not simply substitute for CP1, but instead bypasses the normal CP1-mediated activation mechanism.

Mutations within the CDEI site affect expression of the *MET16-CYC1-lacZ* reporter gene. The results just described indicated that CP1 was required in trans for activity of the *MET16* UAS. To determine if the CP1 binding site was required in cis, site-directed mutations were made within this element, and the effects of those mutations on CP1 binding and UAS activity were as-

sessed. The CDEI consensus sequence, RTCACRTG (R=purine), has been defined in comparisons of yeast centromeric DNA's (Hieter *et al.* 1985). The CDEI elements in MET gene promoter regions differ slightly from that consensus in showing a strong preference for G at position 6, which occurs within the palindromic core (CACGTG), and in lacking a preference for a purine at position 1. The *MET16* CDEI element has a T at position 1 and a G at position 6 (Figure 5). Mutation *met16-47* inserted the dinucleotide TA into the center of the CACGTG palindrome (creating an *Spe*I site), while *met16-48* inverted the central CG. Both of these mutations were expected to have strong negative effects on CP1 binding. Mutation *met16-33* created an *Spe*I site just upstream of the CDEI site, improving homology to consensus by changing position 1 to a G, and coincidentally altering nucleotides outside of the CDEI element. Mutation *met16-39* is a G to A transition at position 6, a change known to have no effect on centromere CDEI function (Niedenthal, Stoll and Hegemann 1991)

Binding of purified yeast CP1 to the mutated *MET16* promoter fragments was analyzed in quantitative electrophoretic mobility shift assays. The assays measured the equilibrium constant of CP1 binding to the *MET16* DNA probe relative to a reference probe containing the CDEI site of *CEN3* (see Materials and Methods). CP1 binding to the wild-type *MET16* UAS was reduced 1.5-fold relative to *CEN3* (a measured K_{rel} of 0.67). K_{rel} values for the *MET16* CDEI mutants, normalized to wild-type *MET16*, are given in Figure 5. As expected, the *met16-47* and *met16-48* mutations reduced CP1 binding significantly, 170- and 300-fold, respectively. Changing the G at position 6 to A (*met16-39*), a nucleotide found in 6 of 15 centromeric CDEI sites, lowered the

binding constant 16-fold. The *met16-33* UAS had a 15-fold increased K_{rel}, confirming the importance of position 1 in CP1 recognition (Baker, Fitzgerald-Hayes and O'Brien 1989).

CYC1-lacZ reporter constructs containing the mutated *MET16* UAS fragments were tested for β-galactosidase expression. This series of plasmids (pKM11 series) differed from the pRB137 series in that the vector lacked *CYC1* sequences upstream of the *Xho*I cloning site. Removal of this DNA led to a 2.5-fold increase in β-galactosidase activity, but as before, expression was completely dependent on CP1 (Table 7). All CDEI site mutations affected the levels of β-galactosidase induced upon methionine limitation. The *met16-47* and *met16-48* mutations resulted in complete loss of activity, while the *met16-39* UAS drove β-galactosidase expression to only 14% of the wild-type level. The correlation between the affinity of these mutated promoter elements for CP1 and their ability to activate expression of the reporter gene in response to methionine limitation strongly suggested that CP1 acted through the CDEI site. In the case of *met16-33*, CP1 binding affinity did not correlate with UAS activity; binding was increased 15-fold, while β-galactosidase expression was reduced about 5-fold (Figure 5, Table 7). Significantly, the *met16-33* mutation changed nucleotides both within and outside of the CDEI site. Analysis of a minimal UAS element derived from the *MET25* promoter has shown that a CDEI site and the 10 bp adjacent to it are essential (Thomas, Jacquemin and Surdin-Kerjan 1992). The results with the *met16-33* mutant may indicate that the same is true for *MET16*, *i.e.*, a critical cis-acting element occurs immediately adjacent to the CDEI element.

Domain-swap experiments. The data presented so far argue that

TABLE 7

Effects of CDEI mutations on expression of the *MET16-CYC1-lacZ* reporter gene

	β-galactosidase activity[a]	
Allele	1.0 mM Met	0.05 mM Met
Wild-type[b]	0.19 ± 0.16 (20)	87.7 ± 25 (23)
met16-47	0.08 ± 0.06 (3)	0.21 ± 0.20 (3)
met16-48	0.10 ± 0.03 (7)	0.16 ± 0.05 (7)
met16-39	0.13 ± 0.04 (8)	12.7 ± 5.5 (8)
met16-33	0.18 ± 0.17 (5)	16.4 ± 2.0 (5)

[a]As in Table 2; host strain was K23-9A.

[b]β-galactosidase activities for the wild-type allele (pKM11-1) in the *cep1* genetic background were 0.11 ± 0.05 (4) under repressing conditions, and 0.17 ± 0.03 (3) under derepressing conditions.

DNA binding is required for CP1 to mediate its role in transcription. However, it has also been proposed that CP1's role in methionine metabolism is fulfilled by a non-DNA bound form (Mellor *et al.* 1991). To demonstrate a direct interaction between CP1 and the *MET16* CDEI site, I investigated whether the *met16-48* mutation could be suppressed by a version of CP1 engineered to specifically recognize this mutated site. Plasmid pRS425CBFAP4 encodes a chimeric version of CP1 (CP1-AP4) in which the CP1 DNA-binding domain has been replaced with the homologous region of the b-HLH protein AP4 (Dang *et al.* 1992). This plasmid is unable to confer a Met^+ phenotype upon the *cep1* strain, probably because the AP4 basic region directs binding to the sequence CAGCTG (the *met16-48* sequence) and not to CDEI sites (Dang *et al.* 1992). A related plasmid pRS425CBFAP4R, encodes a chimeric protein (CP1-AP4R), containing a single amino acid substitution within the AP4 basic region; this mutation allows the chimera to recognize CACGTG and to rescue *cep1* methionine prototrophy (Dang *et al.* 1992). These two plasmids were tested for their ability to reactivate expression of various reporter genes in the *cep1* mutant. Strain K23-9B was transformed with various combinations of reporter and CP1 expression plasmids, and β-galactosidase activities measured as before. Plasmid YEp351 (Hill *et al.* 1986b), which lacks a copy of the *CEP1* gene, was used as a negative control and did not restore activity to any of the reporter genes in the *cep1* strain (Table 8). As expected, the *CEP1*-containing plasmids pDM28 (high-copy) and pDR28ARS (single copy), both restored methionine-responsive activity to the reporter gene carrying the wild-type *MET16* element, but not to reporter genes carrying either the *met16-47* (CACTAGTG) or *met16-48* (CAGCTG) mutations. Consis-

TABLE 8

Domain swap experiments: β-galactosidase activities of various combinations of CP1 expression constructs and reporter genes

Expression Plasmid[b]	[Met][c]	β-galactosidase activity[a] Wild-type (CACGTG)	*met16-47* (CACTAGTG)	*met16-48* (CAGCTG)
YEp351	1.0 mM	0.1 ± 0.12	0.12 ± 0.09	0.11 ± 0.06
	0.05 mM	0.13 ± 0.06	0.12 ± 0.10	0.09 ± 0.06
pDM28	1.0 mM	0.18 ± 0.04	0.15 ± 0.04	0.17 ± 0.03
	0.05 mM	97.5 ± 8.0	0.17 ± 0.03	0.42 ± 0.13
pDR28ARS	1.0 mM	0.80 ± 0.84	0.11 ± 0.08	0.19 ± 0.04
	0.05 mM	130.6 ± 39.7	0.16 ± 0.07	0.25 ± 0.04
pRS425CBFAP4	1.0 mM	0.12 ± 0.08	0.14 ± 0.02	0.14 ± 0.09
	0.05 mM	0.14 ± 0.15	0.14 ± 0.07	0.13 ± 0.08
pRS425CBFAP4R	1.0 mM	0.25 ± 0.03	0.17 ± 0.01	0.16 ± 0.04
	0.05 mM	148.7 ± 16.9	0.33 ± 0.05	0.35 ± 0.08

[a]Cultures were grown to an O.D.$_{600} \geq 2.5$ before β-galactosidase activity was measured in whole cells (Rose, Winston and Hieter 1990). β-galactosidase activities in Miller units are reported as the mean ± standard deviation with the number of determinations in parentheses. The *cep1* strain K23-9B, served as host.

[b]All expression plasmids contained the *LEU2* selectable marker. YEp351 (Hill *et al.* 1986b), pDR28ARS (Masison, O'Connell and Baker 1993), and pRS425CBFAP4 andpRS425CBFAP4R (Dang *et al.* 1992) have all been described.

[c]In synthetic complete medium lacking uracil and leucine.

tent with a requirement for specific recognition of the CDEI site, the plasmid encoding the CDEI-binding chimera (pRS425CBFAP4R), also restored expression to the wild-type *MET16* reporter gene, while the plasmid encoding the AP4-site specific chimera (pRS425CBFAP4) did not. Contrary to predictions the CP1-AP4 chimera failed to reactivate expression of the *met16-48* reporter gene even though this reporter contains an AP4 binding site. Cell extracts were prepared from a strain containing pRS425CBFAP4 and analyzed by gel shift analysis. Specific binding to the *met16-48* (AP4) promoter was not detected (data not shown), indicating either poor expression of the chimera or a failure to recognize the AP4 site. This negative result notwithstanding, the abilities of these plasmid-encoded proteins to recognize CDEI sites was found to correlate perfectly with their abilities to restore expression to the wild-type reporter gene and to rescue the methionine prototrophy. Both proteins which specifically bind CDEI sites (CP1 and CP1-AP4R) restored methionine-independent growth and *MET16* reporter gene expression to the *cep1* mutant while the CP1-AP4 chimera was disabled for all measurable activities.

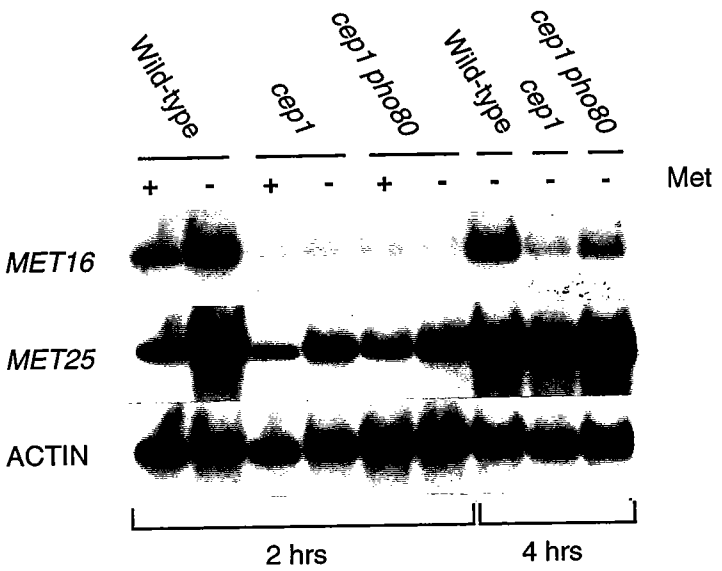
Regulated expression of the chromosomal *MET16* gene requires CP1 and an intact CDEI site. Analysis of the *MET16-CYC1-lacZ* reporter genes allowed indentification of a region of the *MET16* 5'-flanking DNA containing the sequences necessary for methionine-responsive regulation and identification of the CDEI site as an essential cis-acting element. Next, I wanted to extend these observations to the *MET16* gene in its native chromosomal context. Northern analysis was used to measure steady state *MET16* mRNA levels during growth in the presence of methionine (basal expression) or at times following a shift into media lacking methionine (induced expression). To

control for loading and mRNA integrity, Northern blots were probed to detect the constitutively expressed *ACT1* (actin) message. The *cep1* strains were found to contain lower steady-state levels of actin mRNA than wild-type cells grown under similar conditions (see legend to Figure 6). Other groups have not reported this phenomenon (Mellor *et al.* 1991; Thomas, Jacquemin and Surdin-Kerjan, 1992); possibly it is specific to the conditions of my experimental protocol. To avoid this bias, the cumulative data quantitated in Figure 6B have been expressed relative to total RNA. In other cases (Figures 8, 9, and 10), where fewer repetitions were performed, the data were standardized to the internal actin "control". In these cases, *MET16* (and other individual) mRNA's in the *cep1* strain may be overestimated.

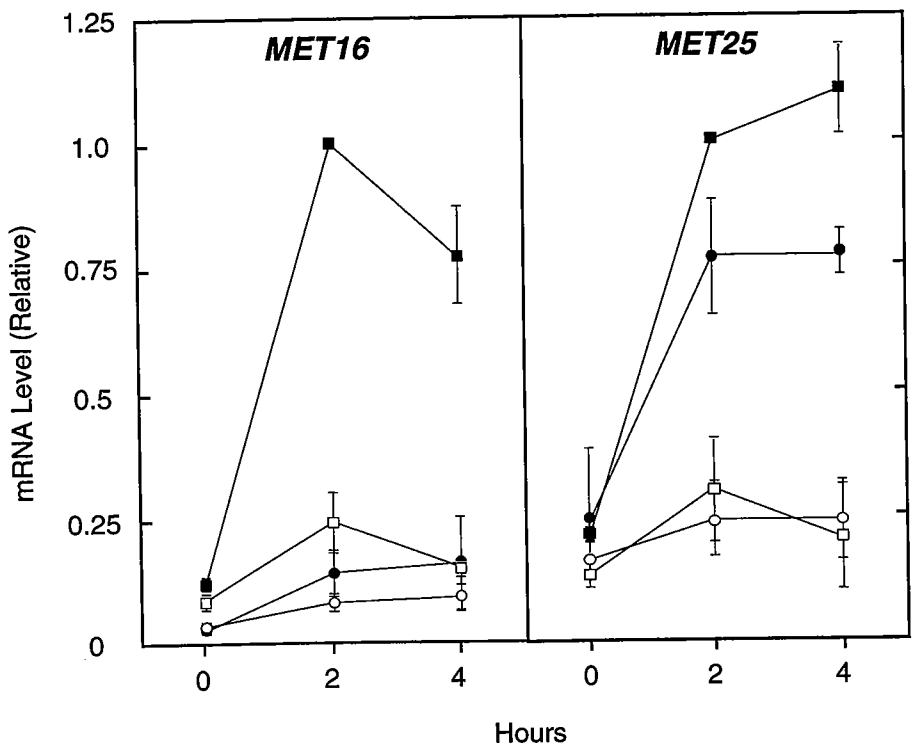
A representative blot is shown in Figure 6A and the quantitated data in Figure 6B. For reference, *MET25* mRNA was also analyzed. Methionine starvation led to a coordinate increase in *MET16* and *MET25* message levels (Figure 6B). Maximum *MET16* message levels, which were reached within 2 h following the media shift, were increased 10-fold over basal levels; thereafter, *MET16* mRNA levels slowly declined. Consistent with earlier observations, the *cep1* mutant did not exhibit the large increase in the level of *MET16* mRNA upon methionine limitation; however, a low level of *MET16* transcript was detected. Basal and derepressed *MET16* message levels were reduced 2.5-fold and 7-fold respectively in the *cep1* strain. Given the total CP1-dependence of the reporter gene, it was surprising to detect *MET16* message in the *cep1* strain. Apparently, the native promoter can partially compensate for the lack of CP1, whereas the reporter gene can not. The *cep1* defect was specific to *MET16*, as expression of *MET25* was largely unaltered in the mutant.

FIGURE 6.—Northern blot analysis of *MET16* RNA. Isogenic strains K23-9A (wild-type), K23-9B (*cep1*), and K23-9D (*cep1 pho80*) were pregrown in methionine-containing (1.0 mM) medium before being shifted into medium lacking methionine or, as the control, back into fresh methionine-containing medium. At the indicated times, samples were collected and the total RNA extracted and analyzed by Northern blotting. *MET16*, *MET25*, and actin RNA's were detected sequentially after stripping the membrane. (A) A composite of radiographic images from a single experiment is shown to illustrate the typical result at 2 and 4 h time points in all three strains. (B) *MET16* and *MET25* message levels in the wild-type and *cep1* strains were quantitated, combining data from eight experiments with an average of five independent determinations for each data point. Values are expressed relative to total RNA and are normalized to the level of the corresponding transcript in the methionine-starved wild-type strain at the two hour time point. □, wild-type $^{+}$Met; ■, wild-type $^{-}$Met; ○, *cep1* $^{+}$Met; ●, *cep1* $^{-}$Met. The normalized values for actin RNA were (mean ± s.d. with the number of determinations in parentheses): wild-type $^{+}$Met, 1.01 ± 0.19 (23); wild-type $^{-}$Met, 1.09 ± 0.18 (25); *cep1* $^{+}$Met, 0.81 ± 0.24 (23); *cep1* $^{-}$Met 0.74 ± 0.19 (25).

A


Wild-type
cep1
cep1 pho80
Wild-type
cep1
cep1 pho80
+ - + - + - - - - Met
MET16
MET25
ACTIN
2 hrs
4 hrs

B


MET16
MET25
1.25
1.0
0.75
0.5
0.25
0
mRNA Level (Relative)
0
2
4
0
2
4
Hours

Due to the longer generation time of *cep1* mutants (Baker and Masison 1990), it was possible that the observed decrease in *MET16* expression in the *cep1* strain was attributable to altered kinetics of induction. This seems unlikely as the kinetics of *MET25* induction were unaffected in the mutant (Figure 6B). Furthermore time course experiments revealed that from as early as 20 min following the media shift to as late as 8 h, *cep1* strains never fully derepressed *MET16* (data not shown).

MET16 expression was also examined in the *cep1 pho80* suppressor strain. Figure 6A shows the typical result: a modest increase in the level of *MET16* mRNA was reproducibly detected in the *cep1 pho80* strain grown under methionine limitation. In experiments in which *cep1* and *cep1 pho80* strains were directly compared, the *cep1 pho80* strain expressed, on the average, 2.4-fold more *MET16* message (measured at 4 h) than that observed in the *cep1* strain (Figure 6A). However, if the *MET16* RNA levels in the *cep1* and *cep1 pho80* strains are normalized to actin message levels, the *cep1 pho80* strain appears to contain only slightly (1.3-fold) more *MET16* RNA than the *cep1* strain. If the latter value (1.3-fold) more accurately reflects the difference in *MET16* message levels between these two strains, it would appear the *pho80* mutation has only a small effect on expression of the endogenous gene. It is possible that *MET16* message levels continue to rise in the suppressor strain, but experiments were systematically terminated after 4 h because the *cep1* mutant ceases to divide shortly thereafter.

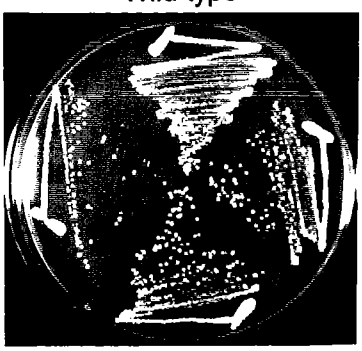
To determine if the CDEI mutations which abolished expression of the *MET16-CYC1-lacZ* gene also affected expression of the chromosomal *MET16* gene, the native copy of *MET16* was replacedwith versions carrying the

met16-47 (CACtaGTG) and *met16-48* (CAgcTG) promoter mutations (see Materials and Methods). The resulting isogenic strains, differing only in sequence within the CDEI element of the *MET16* promoter, were analyzed for their ability to express *MET16* and to grow in the absence of methionine. In comparison to the wild-type strain, both mutant strains grew poorly on media lacking methionine (Figure 7). The *met16-48* mutant grew better than the *met16-47* mutant, suggesting that the former mutation was less deleterious to *MET16* expression. To analyze expression of *MET16* in these strains, cells were shifted into media lacking methionine and message levels measured as before. Despite the fact that both mutations completely abolished expression of the *MET16* reporter gene, neither completely inactivated expression of the native gene (Figure 8). Importantly, the *met16-47* mutation was at least as severe in its effect on *MET16* expression as the *cep1* mutation, confirming that the cis- and trans-acting mutations have equivalent effects on *MET16* expression. As expected from its growth phenotype, the *met16-48* mutation was slightly less severe in its effect than the *met16-47* mutation. When starved for methionine, the *met16-48* strain expressed roughly one third the level of *MET16* message as the wild-type strain. The relatively small difference in *MET16* message levels measured between the *met16-47* and *met16-48* strains translated into a significant difference in their methionine-free growth phenotypes, similar to the observation made for *cep1* and *cep1 pho80* suppressor strains. Interestingly, the *met16-47* and *met16-48* mutations appeared to affect expression of *MET25*. The wild-type and *cep1* strains expressed similar levels of *MET25* message, while *MET25* RNA levels in the *met16-47* and *met16-48* mutants were substantially higher. The enhanced *MET25* response in these

FIGURE 7.—Methionine-free growth phenotypes of strains carrying *met16* promoter mutations. Isogenic strains K23-9A (wild-type), K23-9B (*cep1*), K55-M47 (*met16-47*), and K56-M48 (*met16-48*) were streaked on synthetic complete medium containing or lacking methionine and grown at 30°C for 3 days.

Wild-type

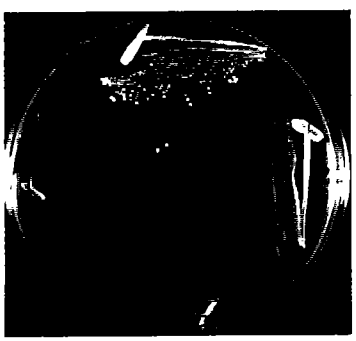
cep1



met16-48

met16-47

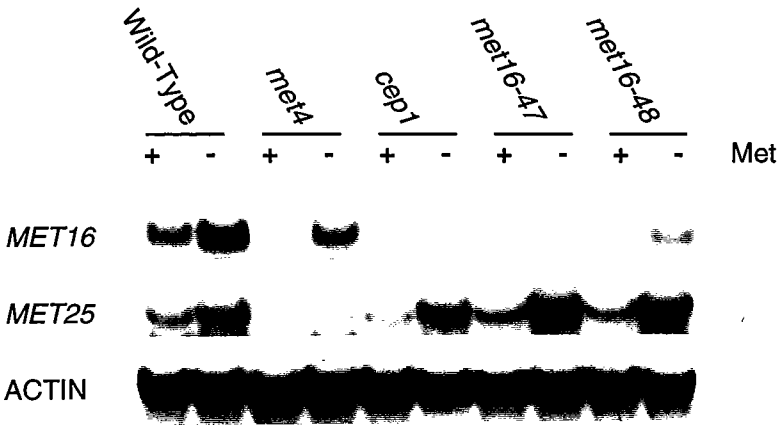
Complete



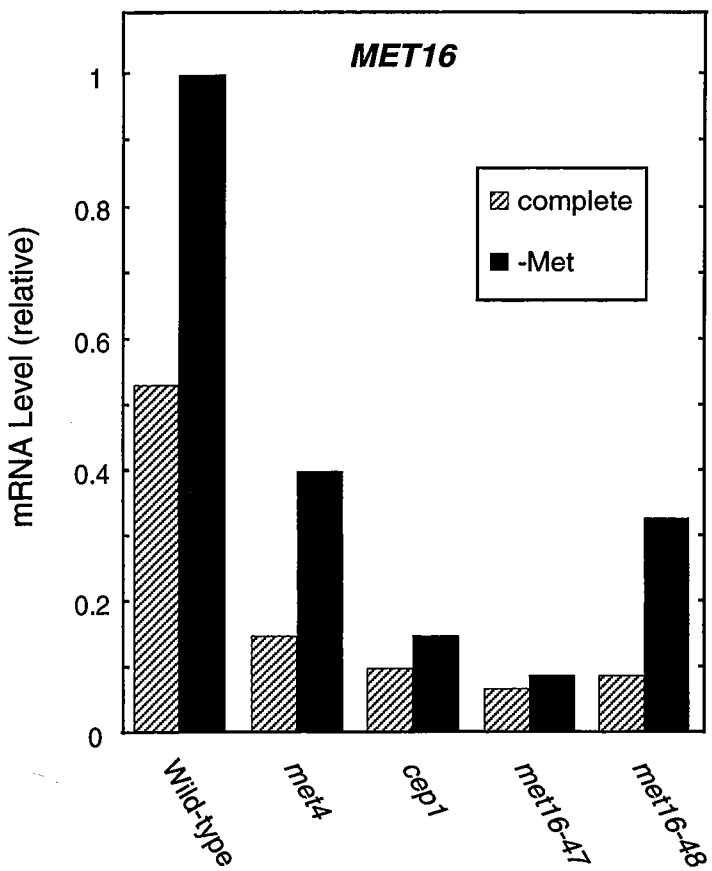
-Met

FIGURE 8.—Effect of *MET16* promoter mutations on *MET16* and *MET25* mRNA levels. Strains K23-9A (wild-type), K23-9B (*cep1*), K55-M47 (*met16-47*), K55-M48 (*met16-48*), and CD107 (*met4*) were subject to a nutritional (methionine) downshift. Total cellular RNA was extracted from samples collected 2 hours following the shift and analyzed by Northern blotting. Shown are: (A) a composite of radiographic images of the blot probed successively for *MET16*, *MET25* and actin; and (B) the quantitation of *MET16* RNA levels. The data were corrected for recovery of actin RNA and normalized to the level of message present in the methionine-starved wild-type strain.

A


Wild-Type
met4
cep1
met16-47
met16-48
+ - + - + - + - + -
Met
MET16
MET25
ACTIN

B


MET16
mRNA Level (relative)
1
0.8
0.6
0.4
0.2
0
complete
-Met
Wild-type
met4
cep1
met16-47
met16-48

½ x 5

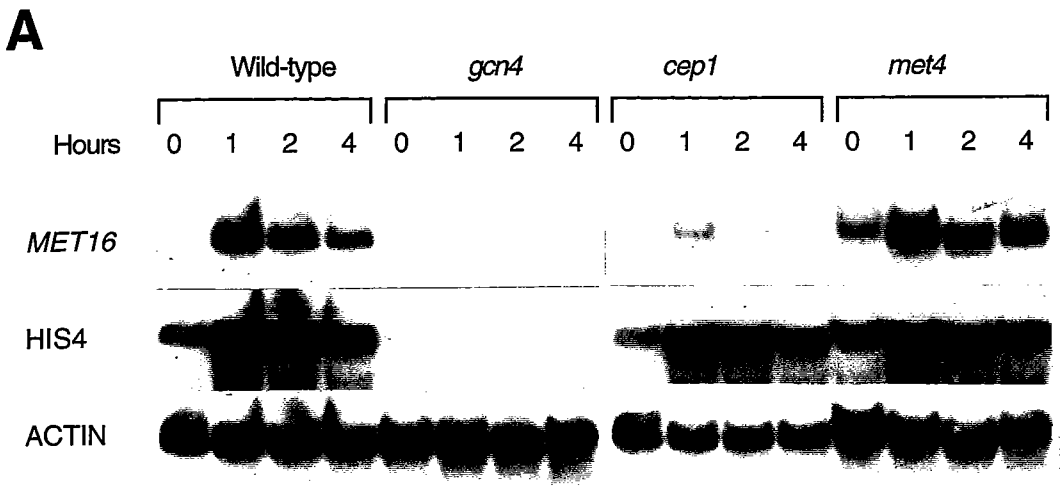
strains was observed consistently and was probably due to activation by the general control pathway (see below).

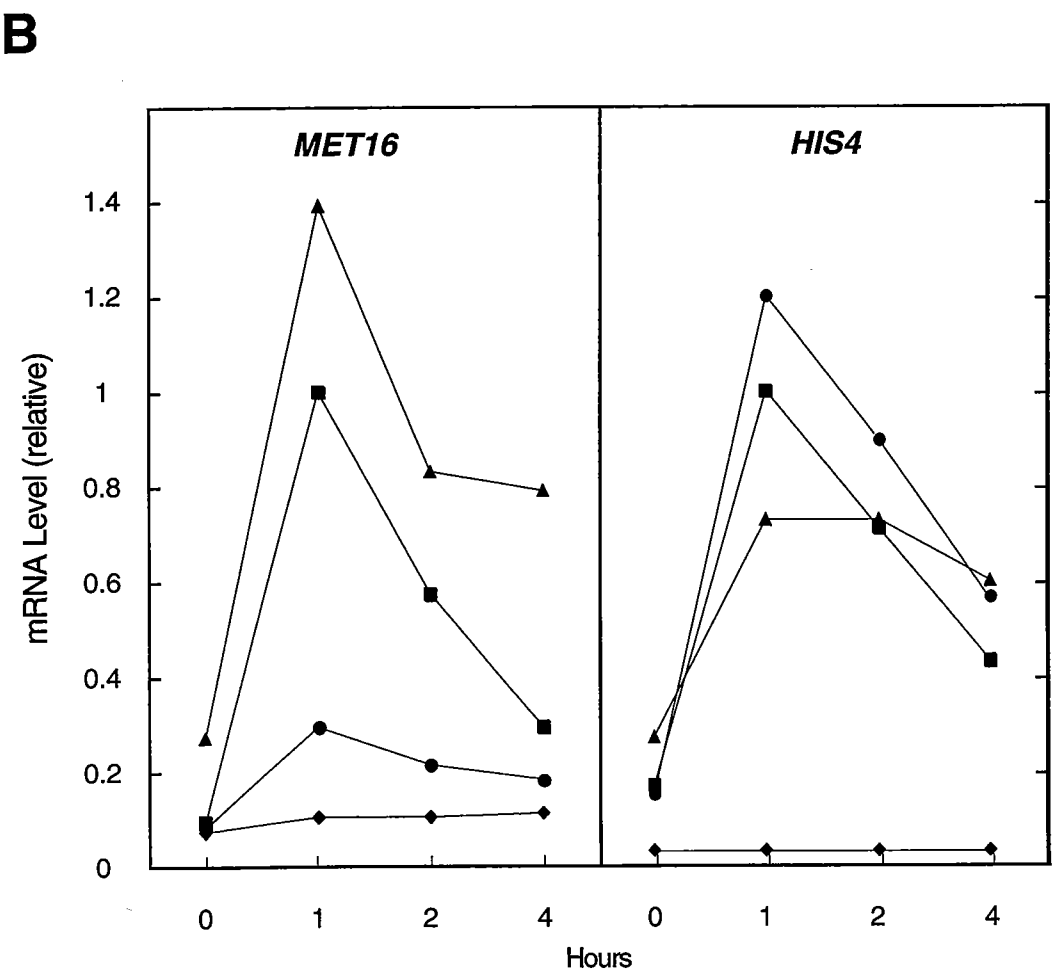
Transcription of *MET16* is regulated by a CP1-dependent general control response. Previous work had indicated that CP1 and MET4 were absolutely required for transcription of *MET16*; no *MET16* mRNA was detected in methionine-starved *cep1* or *met4* strains (Thomas, Jacquemin and Surdin-Kerjan 1992). In the present experiments, weak, CP1-independent transcription of *MET16* was consistently detected. To determine if this discrepancy was simply due to increased sensitivity or if it reflected a more fundamental difference in strains, media, or induction conditions, the *MET4* dependency of *MET16* transcription was analyzed under my experimental conditions. Surprisingly, substantial *MET16* RNA was detected in the *met4* mutant, more than in the *cep1* mutant (Figure 8). Consistent with the earlier findings, the expression of *MET25* was completely dependent on MET4. These results suggested that under these experimental conditions, a second regulatory mechanism was operating at the *MET16* but not at the *MET25* promoter.

A distinguishing feature of the *MET16* 5'-flanking region is the presence of a consensus GCN4-binding site, an element involved in coordinating the expression of many amino acid biosynthetic genes. To determine if *MET16* was regulated by GCN4 and if so what role CP1 might play in this response, the expression of *MET16* was analyzed under growth conditions which trigger general control activation. Cells were pregrown in synthetic media lacking all aromatic amino acids, before being starved for tryptophan by addition of 5-methyltryptophan (5-MT). RNA was prepared from samples taken at various times thereafter and Northern analysis performed. As

shown in Figure 9, *MET16* message levels were rapidly and dramatically elevated in response to tryptophan starvation. Within one hour following addition of 5-MT, *MET16* was fully induced, and thereafter message levels declined. As a control, the blot was reprobed for transcripts of *HIS4*, a gene known to be regulated by general control (Jones and Fink 1982). The expression pattern of *HIS4* was indistinguishable from that of *MET16*. Induction of both genes was due to the general control pathway as demonstrated by the lack of induction of both *MET16* and *HIS4* in the *gcn4* mutant (Figure 9). The ability of 5-MT to induce *MET16* expression was attenuated in the *cep1* mutant; only a 2.5-fold induction was observed, compared with the 10-fold effect seen in the wild-type strain (Figure 9). The induction of HIS4 message was largely unaffected in the *cep1* strain; thus, CP1 was involved specifically in the general control response of *MET16* and did not act generally to facilitate GCN4 function. To determine if MET4 participated in general control of *MET16*, the 5-MT response was examined in a *met4* mutant. Induction of *MET16* transcription was unaffected (Figure 9); thus, the GCN4- and MET4-dependent activation mechanisms appeared to be distinct. If any relationship between the two mechanisms exists, it would be a negative one, since the general control response of *MET16* was more robust in the *met4* strain than in the wild-type strain. I conclude that *MET16* is activated by two mechanisms, one MET4-dependent (pathway specific) and the other GCN4-dependent (general control). To assess the CP1-dependence of pathway-specific regulation in the absence of the general control response, *MET16* expression was examined in *gcn4* and *cep1 gcn4* strains. Isogenic strains, differing only at the *CEP1* and *GCN4* loci, were pregrown under repressing conditions, and the stocks divided among

FIGURE 9.—General control of *MET16* expression. Strains F113 (wild-type), F212 (*gcn4*), K23-9B (*cep1*), and CD107 (*met4*) were grown in synthetic complete medium lacking aromatic amino acids. From each culture an initial sample was removed and the remaining portion adjusted to 1 mM 5-methyltryptophan after which additional samples were removed at the times indicated. Total cellular RNA was extracted from the samples and analyzed by Northern blotting. (A) A composite of radiographic images of the blot probed successively for *MET16*, *HIS4*, and actin mRNA. (B) Relative message level plotted vs. time for *MET16* and *HIS4* mRNA's. The values were corrected for recovery of actin RNA and normalized to the level of the corresponding transcript in the wild-type strain at the 1 hour time point. ■, wild-type; ◆, *gcn4*; ●, *cep1*; ▲, *met4*.

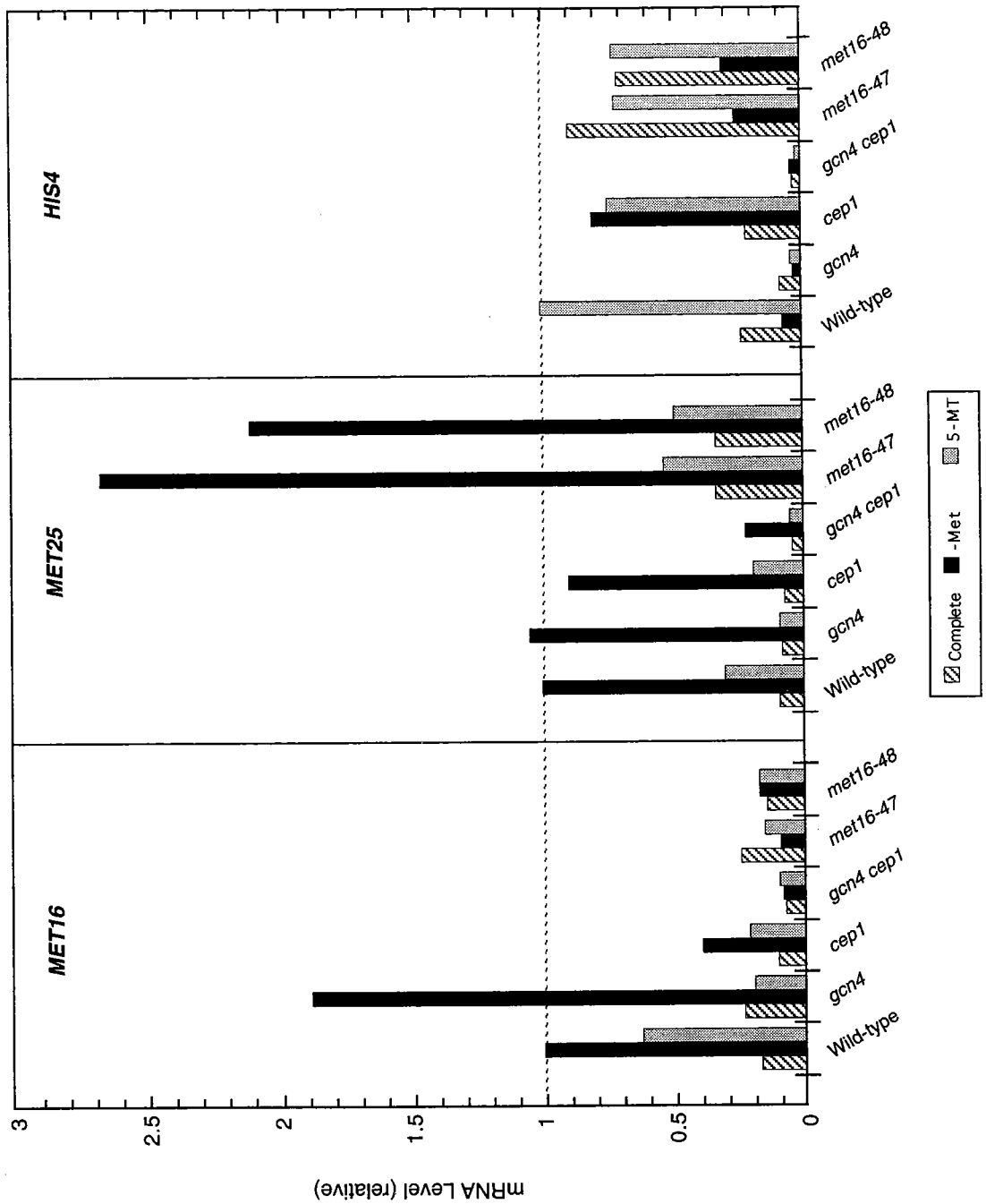

A
Wild-type
gcn4
cep1
met4
Hours
0 1 2 4 0 1 2 4 0 1 2 4 0 1 2 4
MET16
HIS4
ACTIN


B
MET16
HIS4
mRNA Level (relative)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 4 0 1 2 4
Hours

three individual cultures. One culture served as the uninduced control, one was limited for methionine, and one was starved for aromatic amino acids then exposed to 5-MT. Northern analysis revealed that, as before, *MET16* message levels were induced in the wild-type strain by either methionine starvation or 5-MT treatment (Figure 10). In the *gcn4* mutant, *MET16* was not activated by 5-MT, but the response to methionine limitation remained intact, demonstrating that GCN4 was not required for the pathway-specific response. Indeed, much like the general control response in the *met4* mutant, the pathway-specific response in the *gcn4* mutant was enhanced. Most, if not all, of the *MET16* mRNA in the *cep1* strain induced by methionine limitation was due to a CP1-independent component of the general control response, as it was absent in the *cep1 gcn4* double mutant grown under identical conditions. In fact, the *cep1 gcn4* double mutant exhibited the lowest *MET16* message levels under all growth conditions. Thus, CP1 participates to different extents in the two regulatory mechanisms; the MET4-dependent mechanism has an absolute requirement for CP1, while the GCN4-dependent mechanism functions weakly in its absence. The direct involvement of CP1 in both regulatory mechanisms was confirmed by measuring *MET16* expression in the strains carrying the *MET16* promoter mutations; both activation pathways were disabled by the cis-acting *met16-47* and *met16-48* mutations (Figure 10).

As a means to confirm independently that a general control response was triggered in the methionine-starved *cep1* strain, *HIS4* message levels were examined (Figure 10). All strains responded to 5-MT as predicted; *HIS4* expression was activated in every circumstance except in *gcn4* strains. The response of *HIS4* expression to methionine limitation revealed an interesting

FIGURE 10.—General and pathway-specific control of *MET16* expression. Strains F113 (wild-type), F212 (*gcn4*), K64-T1 (*cep1*), K63-T1 (*gcn4 cep1*), K55-M47 (*met16-47*), and K56-M48 (*met16-48*) were pregrown in synthetic complete medium containing 1 mM methionine. Each culture was divided into three aliquots, one of which was immediately used for RNA isolation. The second sample of cells was starved for methionine for 2 h. The third sample was shifted to complete medium lacking aromatic amino acids then induced with 5-methyltryptophan (1 mM) for 1 h. RNA was isolated and analyzed by Northern blotting. The graph shows the levels of *MET16*, *MET25*, and *HIS4* messages in each strain under the specified growth conditions. The data were first corrected for recovery of actin mRNA, then normalized to the level of the corresponding transcript in the methionine-starved wild-type strain (*MET16* and *MET25*) or to that in the 5-MT-treated wild-type strain (*HIS4*).


mRNA Level (relative)
0
0.5
1
1.5
2
2.5
3
MET16
MET25
HIS4
Wild-type
gcn4
cep1
gcn4 cep1
met16-47
met16-48
Complete
-Met
5-MT

relationship; strains exhibiting the weaker methionine-free growth phenotypes possessed the highest *HIS4* message levels. While *HIS4* expression was not activated in the methionine-starved wild-type strain, the auxotrophic *cep1* mutant exhibited a large increase in steady state message levels when grown under identical conditions. This response was mediated by GCN4, as it was absent in the *cep1 gcn4* double mutant. The *met16-47* and *met16-48* mutants, which grow poorly in the absence of methionine (methionine bradytrophic), also exhibited high levels of *HIS4* mRNA. [These strains also possessed high levels of *HIS4* message when grown in complete media, but the basis for this effect and its relationship to general control are presently unclear.] These results confirm that a general control response is activated in the *cep1* mutant by methionine starvation and are consistent with the residual level of *MET16* expression in the methionine-starved *cep1* mutant being due to a GCN4-dependent mechanism.

The CP1-dependence of *MET25* expression was also examined in the experiment presented in Figure 10, and the results provide a reasonable explanation for disparate findings reported in the literature. When expression of MET25 in the wild-type and *cep1* strains was compared, it appeared that the *cep1* mutation had little effect, consistent with the conclusions of Mellor *et al.* (1991). However, if interference from GCN4 was eliminated (compare the *gcn4* and *cep1 gcn4* strains), the *cep1* mutation led to a 5-fold reduction in message levels, similar to the observation made by Thomas, Jacquemin and Surdin-Kerjan (1992). Like *MET16*, the pathway-specific response of *MET25* is strongly dependent on CP1, but in contrast to *MET16*, the general control response of *MET25* is largely CP1-independent. Also, *MET25* expression was

poorly induced by 5-MT treatment, perhaps reflecting an inability of GCN4 to completely overcome AdoMet-mediated repression. Significantly, the large GCN4-dependent increase in *MET25* message levels observed in the methionine-starved *cep1* strain was elicited under conditions in which AdoMet repression should be relieved. The extraordinarily high *MET25* message levels in the methionine-starved *met16-47* and *met16-48* strains (Figures 8 and 10) probably reflect the additive effects of the general and specific control systems. That is, these strains are *CEP1*$^+$ but are methionine bradytrophs due to the *MET16* promoter mutations; growth in the absence of methionine should trigger a full MET4-dependent response in addition to a GCN4-dependent response.

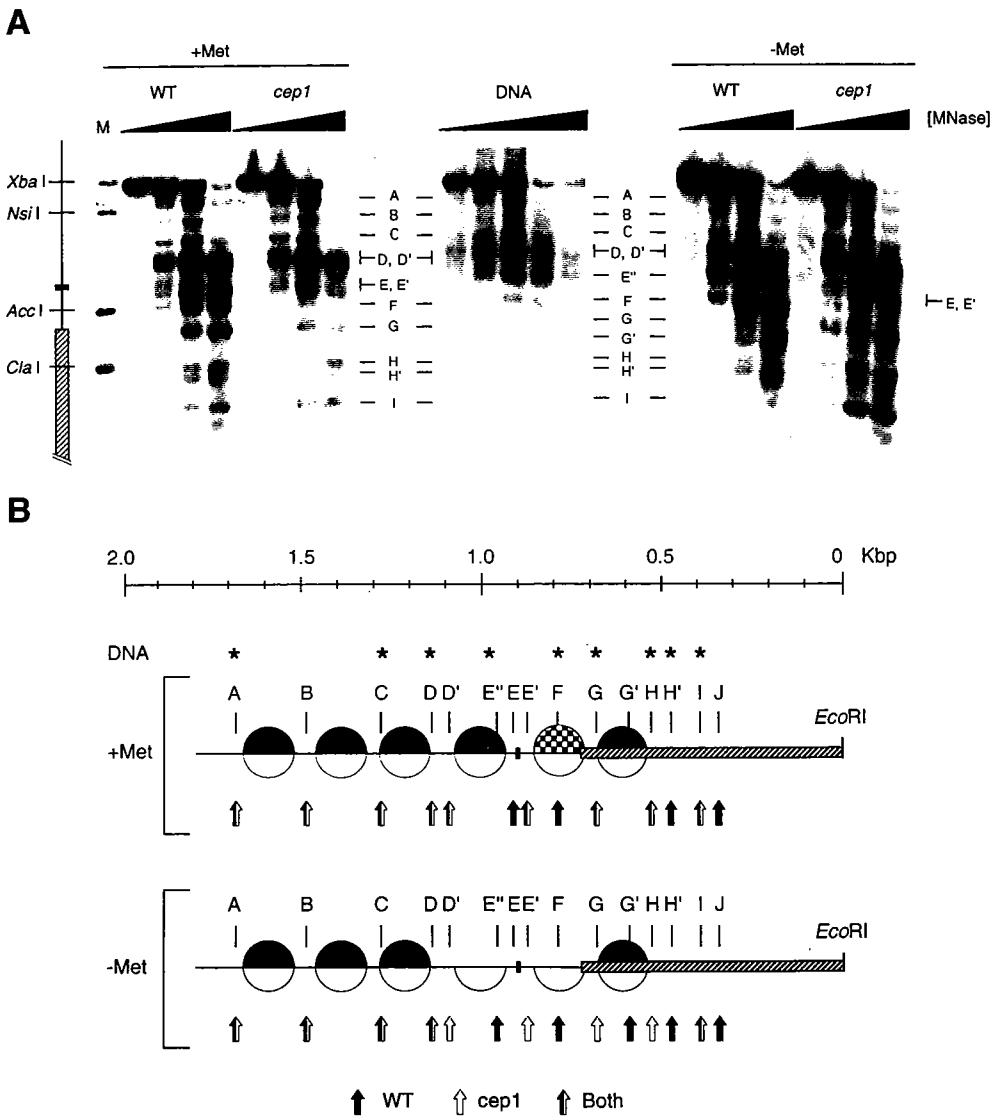
Disruption of *CEP1* leads to localized changes in the chromatin structure of the *MET16* promoter. By traditional criteria, CP1 appears to lack an autonomous transcription activation domain. It has been suggested that CP1 somehow modulates chromatin structure to create an "active" configuration (Mellor *et al.* 1990). Consistent with this idea, strains which lack CP1 exhibit changes in the chromatin structure of CDEI-containing genes such as *TRP1* (Mellor *et al.* 1990). Since expression of *MET16* is strongly dependent on CP1, it was of interest to determine if the chromatin structure of the *MET16* locus differed in *cep1* and wild-type cells.

Micrococcal nuclease (MNase) was used to probe the chromatin structure of *MET16*. Chromatin in spheroplasts of cells grown under repressing or derepressing conditions was partially digested with MNase and the cleavage sites mapped by indirect end-labeling (Wu 1980) For the most part, the *MET16* chromatin of *cep1* cells possessed the same preferred MNase cleavage

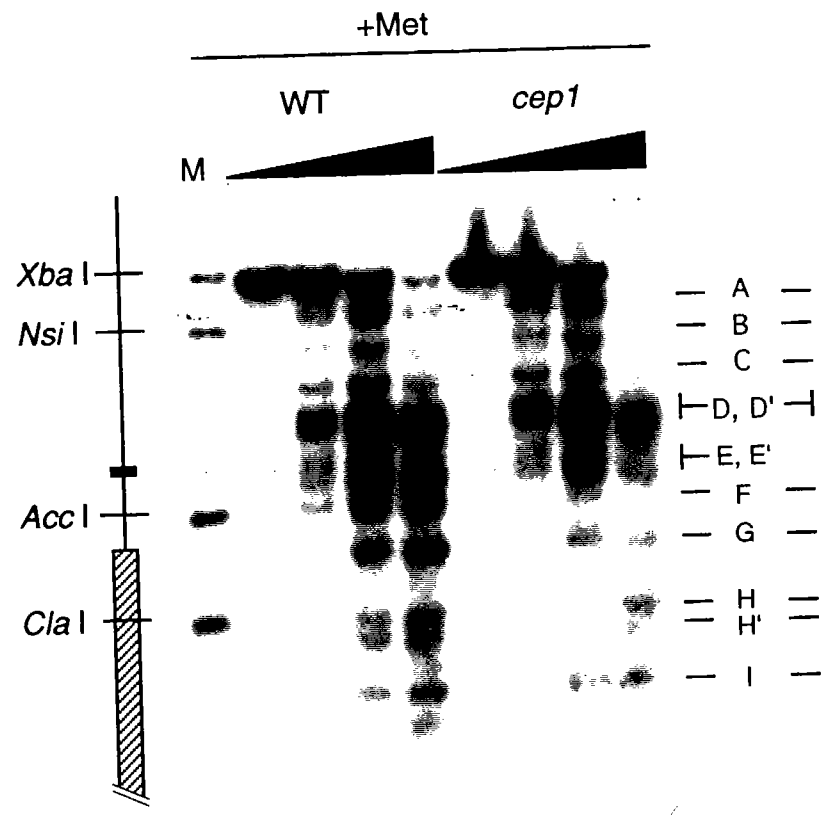
sites as the chromatin of wild-type cells (Figure 11A). In particular, a pattern of strong cleavages spaced at a fairly regular 200-bp interval extending from 950 bp upstream of the initiator ATG to 200 bp upstream of the CDEI site was detected. Although these sites (designated A, B, C, and D) were present in naked DNA, their cleavage was enhanced in chromatin. This same nuclease cleavage pattern was present in both *cep1* and wild-type strains regardless of the growth conditions and therefore was not dependent upon CP1 or upon the transcriptional status of *MET16*. Because naked DNA exhibited a similar pattern of nuclease cleavage, it is difficult to make any inferences concerning the chromatin structure of this region. However, one possible interpretation of the enhanced cleavage pattern observed in chromatin versus naked DNA is that this region is arranged within an array of phased nucleosomes (Figure 11B).

The nuclease digestion pattern of the *MET16* locus in *cep1* chromatin differed from that of naked DNA in several respects. Sites E" and F, sensitive in naked DNA, were protected in *cep1* chromatin, and chromatin-specific sites D', E, and E' appeared, the latter two sites flanking the CDEI element (most apparent in Figure 11C). In wild-type cells, the MNase digestion pattern differed in the region surrounding the CDEI site. Most striking was the accessibility of site F, near the putative TATA box (Thomas, Barbey and Surdin-Kerjan 1990) Site F was protected in *cep1* chromatin but accessible in wild-type chromatin (and naked DNA). Under derepressing conditions (-Met), site F became hypersensitive to MNase digestion in the wild-type strain but remained protected in the *cep1* strain. If it is assumed that these differences are due to positioned nucleosomes, the model shown in Figure 11B can be

FIGURE 11.—Indirect end-label analysis of *MET16* chromatin structure in wild-type and *cep1* cells. Chromatin in strains K23-9A (wild-type) and K23-9B (*cep1*) grown under repressed (+Met) or methionine-starvation (-Met) conditions was analyzed using the indirect end-labeling protocol described in Materials and Methods. The hybridization probe was complementary to sequences adjacent to the *Eco*RI site. (A) A radiographic image of a typical blot is shown with micrococcal nuclease (MNase) cleavage sites identified by uppercase letters. Lanes marked "DNA" contained deproteinized DNA treated with (from left to right) 1.5, 2.9, 5.9, 5.9, 8 8 U/ml MNase. Other lanes contained chromatin treated with (from left to right) 0, 9.2, 37, and 147 U/ml MNase. Lane M contains a mixture of genomic DNA triple digests (*Eco*RI, *Xba*I, and either *Cla*I, *Acc*I, or *Nsi*I). (B) MNase cleavage map and a model of possible nucleosome positions. The *MET16* coding region is depicted by the diagonally-striped bar and the CDEI site by the small solid rectangle. Asterisks denote MNase cleavage sites in naked DNA. The upward-pointing arrows below each map indicate MNase cleavage sites in chromatin. Solid arrows denote cleavages detected in wild-type chromatin, open arrows cleavages in *cep1* chromatin, and half-open arrows cleavages observed for both strains. The semicircles represent nucleosomes and are drawn to scale (146-bp). Solid semicircles represent nucleosomes inferred to be present in the wild-type strain, open semicircles nucleosomes inferred to be present in the *cep1* strain. The checkered semicircle represents a nucleosome in wild-type chromatin that only partially protects the underlying sequence. It is probably present in only a subpopulation of DNA molecules. (C) An enlargement of the repressed chromatin samples from section 11A showing sites D' E and E' (separate page).


A
+Met
-Met
WT
cep1
DNA
M
[MNase]
Xba I
Nsi I
Acc I
Cla I
A
B
C
D, D'
E, E'
F
G
H
H'
I
E"
G'
B
2.0
1.5
1.0
0.5
0 Kbp
DNA
+Met
-Met
A B C D D' E" E E' F G G' H H' I J
EcoRI
WT
cep1
Both

C



drawn. Although speculative, this model suggests that the main difference between the wild-type and *cep1* strain is the presence of a positioned nucleosome adjacent to the CDEI site in the *cep1* strain. In the wild-type strain this nucleosome may be totally absent in a subpopulation of cells or loosely associated with this region in all cells.

Upstream of the CDEI site, no major differences between the wild-type and *cep1* strains were observed in repressed ($^+$Met) chromatin, but protection of site E'' was lost in wild-type chromatin under derepressing conditions ($^-$Met). In fact, the overall digestion pattern of derepressed wild-type chromatin in the CDEI region resembled that of naked DNA (sites D, E'', and F present; sites D', E, and E' absent or reduced). Again, these changes in the chromatin structure are difficult to interpret but the data are consistent with a model in which two nucleosomes flanking the CDEI element are lost from wild-type chromatin uder derepressing conditions (Figure 11B). Downstream of site F, additional differences were observed under conditions of *MET16* derepression ($^-$Met), but only in wild-type chromatin. Cleavage at sites G' and H' was prominent, while sites G, H, and I were protected. Since these changes occurred in the transcribed region, they were probably a direct consequence of transcription.

CHAPTER V

DISCUSSION

Strains carrying a disruption of *CEP1*, the gene encoding the yeast centromere-binding protein CP1, are methionine auxotrophs. Although circumstantial evidence suggests that CP1 is required for expression of methionine biosynthetic genes, direct evidence to support a transcriptional role for CP1 is lacking. The methionine requirement of *cep1* mutants was originally studied genetically through the isolation of suppressors. Three separate lines of evidence support the conclusion that activation of the transcription factor PHO4 in *cep1* null mutants result in suppression of the *cep1* methionine auxotrophy. First, the presence of one or more extrachromosomal copies of *PHO4* in the *cep1* background confer the Met^+ phenotype; the resulting overexpression of *PHO4* presumably upset the balance between PHO4 and its negative regulator PHO80, creating a pool of active transcription factor. Second, mutations which disrupt or eliminate *PHO80* regulation (*e.g.*, $PHO4^c$, *pho80*) also suppressed *cep1* methionine auxotrophy, and a significant portion (about 30%) of independent spontaneously arising suppressors are alleles of *pho80*. Third, disruption of *PHO84*, which results in lowered intracellular P_i levels and constitutive activation of PHO4, suppresses *cep1* auxotrophy. In all cases (except for the weak suppression observed when *PHO4* is carried on a CEN plasmid), suppression correlates with increased rAPase expression indicative of *PHO* regulon derepression. However, suppression appears to be a direct effect of PHO4. Neither *PHO5* (rAPase) nor *PHO84* (phosphate permease), two downstream targets of PHO4, are required for the *PHO4*-dependent suppression. The most straightforward interpretation of these findings is that an active PHO4 transcription factor can functionally substitute for CP1 in regulating methionine biosynthesis.

One condition where *PHO4* activation does not result in suppression is when *cep1* mutants are grown in medium containing derepressing concentrations of P_i. Although rAPase is derepressed, no growth occurs in the absence of methionine. Quantitation of acid phosphatase levels reveals that *cep1* strains grown in complete synthetic media at low P_i concentrations contain rAPase activities higher than isogenic strains carrying a *PHO4^c* mutation or extra plasmid copies of *PHO4*. Thus, if rAPase levels are a reliable measure of PHO4 activity, cells starved for P_i appear to possess the requisite PHO4 activity. It is possible that under certain circumstances rAPase activity fails to accurately reflect PHO4 activity. Cultures of plasmid-bearing cells are heterogeneous with respect to plasmid copy-number; therefore, the rAPase activity determined for a strain grown under selection for the plasmid may underestimate the true activity of the methionine prototrophs. In the case of the surprisingly low rAPase activity present in the *PHO4^c* strain (K22-T8), it is possible that an unlinked mutation affects *PHO5* expression without affecting suppression. Consistent with this idea, when the *cep1 PHO4^c* strain was backcrossed to a *cep1 PHO4$^+$* strain, the diploid gave rise to some Met$^+$ segregants with very high phosphatase activities (data not shown). Alternatively, it is possible that the rAPase activity determined for *cep1* strains grown under derepressing conditions overestimates the actual activity which would be present in cells grown in the absence of methionine. Since *cep1* strains do not grow in the absence of methionine, rAPase activity had to be measured for cells grown in complete medium.

The finding that PHO4 can functionally substitute for CP1 in activating methionine biosynthesis is consistent with the notion that CP1 acts at the

level of transcriptional initiation. PHO4 is known to be a transcriptional activator and probably suppresses the *cep1* methionine requirement by reactivating expression of CP1-dependent *MET* genes. The *pho80* suppressor was tested for its ability to restore *MET16* mRNA levels in a *cep1* strain. As demonstrated for both the *MET16* reporter gene and the endogenous gene, expression was partially restored by the *pho80* mutation. So how is PHO4 activity targeted to the *MET16* promoter? It appears that CP1 and PHO4 have similar DNA-binding specificities. Fisher, Jayaraman and Goding (1991) have shown that PHO4 specifically recognizes CACGTG, the sequence found upstream of *PHO* and *MET* genes and at centromeres. Two such elements within the *PHO5* promoter are protected by PHO4 from nuclease cleavage in vitro (Vogel, Hörz and Hinnen 1989) and are associated with UAS activity in vivo (Rudolph and Hinnen 1987). The similar binding specificities possessed by the two proteins probably stems from the fact that both CP1 and PHO4 are b-HLH proteins with similar basic (DNA-binding) domains (Fisher, Jayaraman and Goding 1991). In fact domain-swap experiments have demonstrated that the basic region of c-Myc, a CACGTG-binding protein, can functionally substitute for the corresponding domains of both CP1 and PHO4 (Dang *et al.* 1992; Fisher, Jayaraman and Goding 1991). Thus, it is likely that both CP1 and PHO4 activate transcription of *MET* genes by binding to the upstream CDEI sites.

CP1 and PHO4 do not possess redundant functions and probably function differently at promoter regions. First of all, PHO4 and CP1 are not interchangeable, otherwise overexpression of *PHO4* should also suppress the *cep1* chromosome-loss phenotype, which it does not (R. Baker unpublished re-

sults). Secondly, *CEP1* overexpression should suppress the Pho$^-$ phenotype of a *pho4* mutant. While lack of mutual suppression could be explained by subtle differences in binding specificity, it could also indicate a fundamental difference in the mechanism by which CP1 and PHO4 activate transcription. Thirdly, CP1, unlike PHO4, appears to lack a transcription activation domain (Thomas, Jacquemin and Surdin-Kerjan 1992; R. Baker, unpublished). Despite these differences, it remains possible that PHO4 literally substitutes for CP1 to activate *MET16* expression via the normal regulatory mechanism. A prediction of this suppression-by-substitution hypothesis is that the activation domain of PHO4 would be dispensable for suppression. To test this hypothesis variants of PHO4 lacking the activation domain were assayed for their ability to suppress the *cep1* methionine requirement. Although DNA-binding proficient (Ogawa and Oshima 1990), three variants of PHO4 which lacked the activation domain were incapable of suppressing the methionine requirement of a *cep1 pho4* strain. One of these alleles (*Δ4-171*) did suppress the methionine requirement of the *cep1* (*PHO4*$^+$)strain, but apparently did so by activating the endogenous wild-type PHO4 protein by titrating the PHO80 repressor. These results indicate that PHO4 utilizes its activation domain to bypass the CP1-dependent activation mechanism. The mechanism by which MET16 transcription is normally regulated probably also relies upon a conventional activation domain, but in this regulatory scheme, that domain is provided by the MET4 and/or GCN4 transcriptional activators.

The *PHO2*-dependence of *cep1* suppression is interesting. While the *PHO2* requirement may be *PHO4*-specific (*e.g.*, stabilization of PHO4 protein binding), it may also reflect the general regulatory role of PHO2 itself. PHO2 is

the same as BAS2, which was originally identified as a factor required for basal level transcription of *HIS4* and probably one or more genes involved in adenine biosynthesis (Arndt, Styles and Fink 1987). *HIS4* transcription is regulated in response to the inorganic phosphate concentration of the growth media and this regulation requires PHO2. Also, Braus *et al.* (1989) have shown that *PHO2* modulates the general control response of TRP4, and they suggest that PHO2 is a general regulator of cellular metabolism in response to phosphate availability. Perhaps it is not coincidental then that *HIS4* and TRP4 enzymes catalyze reactions involving phosphorylated substrates. Methionine biosynthesis requires two phosphorylated intermediates, adenylylsulfate (APS) and phosphoadenylylsulfate (PAPS). PAPS is a general sulfate donor and is the direct substrate of PAPS reductase, the enzyme encoded by *MET16*. The finding that suppression of *cep1* methionine auxotrophy requires *PHO2* may be a consequence of normal *MET16* regulation by *PHO2*.

Several results suggest the existence of regulatory cross-talk between the biochemical pathways utilizing sulfate and phosphate. Under every condition of *PHO* regulon derepression examined—*PHO4*^{c}, *pho80*, *pho84* mutations and low P_i growth conditions—rAPase activity was reduced approximately 2-fold in the *cep1* genetic background (Tables 2 and 3); therefore, *cep1* gene disruption leads not only to a block in sulfate assimilation but also a perturbation in *PHO* gene expression. The *cep1* null mutation was also found to suppress the Pho^{-} phenotype of a *pho84* (phosphate permease) mutant. Since transport is the first step in metabolite utilization, it is well suited as a regulatory point. The mutual suppression of *cep1* and *pho84* may reflect coordination of the two pathways at this step. *PHO84* expression is *PHO2*-dependent,

and if *PHO2* also regulates sulfate permease, this could explain the *PHO2* dependence of *cep1* suppression. None of these results rule out the possibility that *PHO4* itself normally regulates *MET* genes. Activation of PHO4 in a *cep1* genetic background was shown to restore methionine-responsive regulation to both a *MET16* reporter gene and the endogenous gene. It is possible that under certain conditions (i.e.. low phosphate) PHO4 modulates expression of *MET16*. HLH factors are known to form heteromers with other HLH family members (Braun *et al.* 1990; Davis *et al.* 1990; Murre *et al.* 1989), and heteromerization can alter DNA-binding specificity and/or transcriptional activation potential (Benezra *et al.* 1990; Blackwell and Weintraub 1990; Sun and Baltimore 1991). PHO4 might regulate *MET* gene transcription as a heterodimer with CP1. The PHO4 component would provide transcription activation function and the CP1 component would block PHO80 interaction and direct binding to *MET* gene promoters. In *pho4* mutants (which are not methionine auxotrophs), *MET* gene activation could still be effected by the CP1 homodimer, while in *cep1* mutants, PHO4 homodimers would substitute when negative regulation by PHO80 is relieved. Consistent with such a model, the *MET25* UAS identified by Thomas, Cherest and Serdin-Kerjan (1989) contains the sequence AAATGGCACGT which, allowing a one nucleotide insertion, matches the PHO4 binding site UASp1 in the *PHO5* promoter at 10 of 11 positions (Vogel, Hörz and Hinnen 1989). It is currently not clear if this homology has any functional significance, or whether *MET* and *PHO* genes normally utilize common transcription factors.

The role of CP1 in expression of *MET16* was analyzed directly and the results again argue that CP1 acts at the level of transcriptional initiation. An

earlier study had demonstrated that the *cep1* mutation led to an undetectable level of *MET16* message, but the authors did not determine the basis for this effect (Thomas, Jacquemin and Surdin-Kerjan 1992), and thus, it remained uncertain at which step CP1 was acting (transcription initiation, processing, decay, etc.) To address this question, a reporter gene was constructed by placing a fragment from the *MET16* promoter upstream of a *CYC1-lacZ* fusion gene. Like the endogenous *MET16* gene, the reporter gene exhibits strong methionine-responsive regulation which depends upon both CP1 and the integrity of the CDEI site. These results indicate that CP1 acts at the level of the *MET16* promoter and are most consistent with a role in transcriptional initiation. Although I did not directly test the *cep1* mutation for a potential effect on *MET16* message stability, results obtained from analysis of the *MET16* reporter gene indicate that the *cep1* mutation exerts its effect primarily, if not completely, at the level of message synthesis and not decay.

CP1 almost certainly mediates its transcriptional regulatory function by binding to the *MET16* CDEI site. With respect to their effects on expression of the native and reporter gene, the trans-acting *cep1* mutation and the strongest cis-acting CDEI mutation (*met16-48*) are similar. Both mutations completely abolish reporter gene activity and reduce native gene expression approximately 10-fold. Also, there is a positive correlation between CP1 binding to mutated promoter elements and their abilities to activate expression of the *CYC1-lacZ* reporter. Significantly, the *met16-39* mutation has an intermediate effect on both binding and expression. The single exception to the correlation between CP1 binding affinity and reporter gene expression is the *met16-33* mutation. This mutation improves similarity of the *MET16* element to the

CDEI consensus sequence and increases CP1 binding affinity; however, reporter gene expression was reduced. Since the *met16-33* mutation involves changes outside the CACGTG core element, it seems likely that a second important cis-regulatory element either directly abuts or overlaps the CDEI site and that these changes interfere with the ability an important regulatory factor (MET4 ?) to bind this site. A similar arrangement of regulatory elements appears to exist at the *MET25* promoter. Thomas, Jacquemin and Surdin-Kerjan (1992) found that an 18-bp oligonucleotide containing one of the CDEI-sites from the *MET25* promoter was sufficient for normal methionine-responsive regulation and depended on both CP1 and MET4. These results indicate that CP1 binding is necessary but not sufficient for UAS activity and that at least one other regulatory element lies in close proximity to the *MET16* CDEI site.

Other data support the idea that CP1 must bind to promoter CDEI sites to regulate expression of *MET* genes. Using various CP1 chimeras, Dang *et al.* (1992) established a correlation between the ability of these proteins to recognize CDEI sites and their ability to rescue *cep1* methionine auxotrophy. I tested two of these chimeras for their ability to activate expression of the *MET16* reporter gene: CP1-AP4 which neither recognizes CDEI sites nor rescues *cep1* methionine prototrophy, and CP1-AP4R which does recognize CDEI sites and does rescue methionine prototrophy. The results are consistent with those obtained for the methionine-independent growth phenotypes; the CP1-AP4 chimera is inactive, while the CP1-AP4R chimera is similar to native CP1 in its ability to activate the reporter gene. Thus, these results, like those obtained for the CDEI mutations, exhibit a correlation between specific

recognition of CACGTG and ability to activate *MET16* expression. Although these data must be interpreted with caution since nothing is known about the stability of these proteins in yeast, the results are consistent with all other data and strongly argue that specific recognition of CACGTG by CP1 is required for its role in transcription.

A surprising finding of this study was that transcription of *MET16* was regulated by a general control mechanism. Upon cloning *MET16*, Thomas, Jacquemin and Surdin-Kerjan (1992) noted the occurrence of a consensus GCN4 binding site in the promoter region, but observed no gene activation upon arginine-limited growth. The results presented here clearly demonstrate that *MET16* can be activated by the general control system in response to tryptophan starvation. 5-MT treatment activates *MET16* in parallel with *HIS4*, and the transcription of both genes is GCN4-dependent. *MET16*, but not *HIS4*, also requires CP1 to respond maximally to 5-MT; 5-MT treatment elicits a suboptimal response in *cep1* mutants and an undetectable response in strains carrying the *met16-47* and *met16-48* CDEI mutations. Activation of *MET16* by general control did not require *MET4* and thus is distinct from the methionine-specific activation pathway. In fact, the *met4* mutant actually displays increased *MET16* mRNA levels upon 5-MT treatment. Likewise, GCN4 is not required for the MET4-dependent response and actually interferes with it. Thus, downstream of the CP1-dependent step, the activation mechanisms are distinct and each seems to interfere with activation by the other.

Interestingly, activation of the general control pathway upon methionine starvation might explain the slight discrepancy observed between the ex-

pression of the *CYC1-lacZ* reporter gene, which was totally CP1-dependent, and the endogenous *MET16* gene, which always produced low, but reproducible levels of *MET16* mRNA. For measuring reporter activity, cells were grown in the presence of a limiting concentration of methionine (50 μM), while for measuring endogenous *MET16* mRNA levels, a starvation protocol was employed. Conceivably, a full GCN4 response occurs only as cells approach total methionine depletion, a condition apparently not encountered during growth in the presence of 50 μM methionine. The lack of a general control response in cells growing under methionine-limiting but nonstarvation conditions could also explain why Thomas, Jacquemin and Surdin-Kerjan (1992) found *MET16* expression to be totally dependent on MET4. In their experiments, cells were grown in the presence of 0.2 mM homocysteine, and therefore the general control pathway may not have been operative.

MET16 belongs to a group of *MET* genes whose transcription is coordinately regulated in response to methionine availability. While all of these genes contain CDEI sites within their promoters, only *MET16* exhibits a clear requirement for CP1. *MET25* is also a member of this group and, in the present study, its expression was analyzed along with *MET16*. As previously reported, these two genes appear to be coordinately regulated (Thomas, Jacquemin and Surdin-Kerjan 1992). In fact, following a shift into media lacking methionine, *MET16* and *MET25* are induced with identical kinetics. However, in contrast to *MET16*, expression of *MET25* is unaffected by the *cep1* mutation, consistent with the conclusions of one group (Mellor *et al.* 1991), and in apparent conflict with those of a second (Thomas, Jacquemin and Surdin-Kerjan 1992). Interestingly, when the effect of the *cep1* mutation on

MET25 message levels is reexamined in a *gcn4* genetic background, CP1-dependence is clearly detected, and the magnitude of the effect is similar to that reported by Thomas, Jacquemin and Surdin-Kerjan (1992). Thus, my data explain the disparate findings reported in the literature. The apparently normal pathway-specific *MET25* response observed in the *cep1* strain in this study and by Mellor *et al.* (1991) was actually a general control response activated in the *cep1* strain under their (5 μM) and my (no methionine) growth conditions. This response was apparently not triggered under the growth conditions used by Thomas, Jacquemin and Surdin-Kerjan (1992) and thus these authors only observed the pathway-specific response. Thus, *MET25* is also regulated by a *MET4*-dependent, and a *GCN4*-dependent response, but a significant difference between the general control responses of *MET25* and *MET16* is that the *GCN4*-dependent activation of *MET25* is largely CP1-independent. These results most likely explain why earlier experiments failed to detect an effect of the *cep1* mutation on other CDEI-containing *MET* genes.

Regulation of *MET25* transcription by the general control system was unexpected. O-acetylhomoserine sulfhydrylase activity (encoded by *MET25*) does not appear to be regulated by the general control system and the 5'-flanking region of this gene does not contain the GCN4 binding site consensus sequence TGACTC (Kerjan, Cherest and Surdin-Kerjan 1986). However, a closely related sequence occurs at two positions upstream of the *MET25* coding region. The sequence TGACTA is present at position -257 (relative to the translational ATG) and also in opposite orientation at position -239. With respect to the expanded GCN4 consensus (Hinnebusch 1988), the site at -257 matches in 10 of 12 positions (AATGACTAATTA). In addition, the -257 site

is located in similar proximity to a CDEI site as the consensus GCN4 site in the *MET16* promoter (45 and 32 bp downstream, respectively). Results of saturation mutagenesis of a *HIS3* TGACTC element (Hill *et al.* 1986a) would indicate that the sequence at position -257 of the *MET25* promoter would confer only weak activation by GCN4, and indeed *MET25* is only weakly activated in strains grown in complete media containing 5-MT. In contrast, methionine starvation of the *cep1* strain leads to a large general control response. Perhaps GCN4 can not overcome the barrier to *MET25* transcription presented by AdoMet repression. For many amino acid biosynthetic genes, pathway-specific repression mechanisms are known to partially or completely override general control-mediated derepression (Hinnebusch 1988). Thus, GCN4 may only activate *MET25* transcription under conditions where AdoMet-mediated repression is simultaneous relieved, a condition only met in three experimental situations, *i.e.*, in the *cep1*, *met16-47* and *met16-48* strains grown under methionine limitation. In the *cep1* strain, general control entirely compensates for the loss of the pathway-specific response, while in the two strains carrying *MET16* promoter mutations, the enhanced response probably arises from the additive effects of the specific and general control mechanisms. The differential ability of GCN4 to activate *MET16* and *MET25* during growth in the presence of methionine may reflect relative GCN4 binding site affinities.

As a first step towards defining CP1's role at promoter CDEI sites in molecular terms, I analyzed the *cep1* mutation for a possible affect on chromatin structure. A previous study had shown that the *cep1* mutation led to changes in the chromatin structure of centromeres and CDEI-containing promoters (Mellor *et al.* 1990). In the present study, I analayzed the chro-

matin structure of the *MET16* locus by micrococcal nuclease digestion and indirect end-labeling. One possible interpretation of the nuclease digestion data is that under conditions repressive for transcription, the *MET16* locus is packaged within a phased array of nucleosomes with the CDEI site positioned within an internucleosomal linker region. Specifically positioned nucleosomes have also been observed in the promoter regions of the yeast *PHO5* gene (Almer and Hörz 1986), the *BAR1* and *STE6* genes (Shimizu *et al.* 1991), and the *GAL1-GAL10* intergenic region (Fedor, Lue and Kornberg 1988). For the repressed *PHO5* gene it has been shown that one of the PHO4 binding sites is found within a linker region between two nucleosomes.

Comparison of the MNase digestion pattern of wild-type chromatin with that of the *cep1* strain reveals that the overall chromatin structure of the *MET16* locus is nearly the same in the two strains. Thus, loss of CP1 does not lead to a dramatic change in the chromatin structure of this region. In the region around the CDEI site, differences between the strains were apparent. The CDEI site was flanked in chromatin by two hypersensitive sites. While both sites were present in chromatin from the *cep1* strain, the upstream site appeared to be somewhat less accessible, perhaps reflecting a direct effect of CP1 binding or the incorporation of this region into an upstream nucleosome. Additional differences were apparent downstream of the CDEI site. This region was only poorly protected from nuclease cleavage in the wild-type strain, but in the *cep1* strain strong protection was observed. Although other interpretations are possible, these data may reflect the presence of a positioned nucleosome in the *cep1* strain and a somewhat altered association of this nucleosome with the underlying sequence in the wild-type strain. Since this re-

gion contains the putative TATA box and the transcription initiation site (Thomas, Barbey and Surdin-Kerjan 1990) the presence of a stably positioned nucleosome would probably be detrimental to transcription. Thus, at some level CP1 appears involved in modulating chromatin structure, and it is tempting to speculate tht at the *MET16* promoter CP1 may be involved directly or indirectly in inhibiting formation of a nucleosome adjacent to its binding site.

Growth under derepressing conditions brought about further changes in the chromatin structure of the *MET16* promoter, but only in wild-type cells. The region downstream of the CDEI site, weakly protected from cleavage under repressing conditions, became completely accessible. The region immediately upstream of the CDEI site also became accessible to nuclease cleavage. It is possible these changes reflect the complete loss of two nucleosomes flanking the CDEI site. The changes that appear to take place in the chromatin structure of the *MET16* locus upon induction are similar to the changes that take place at the *PHO5* locus. Induction of *PHO5* transcription is accompanied by the loss of four positioned nucleosomes from the promoter: two immediately upstream of a PHO4 binding site and two immediately downstream (Almer *et al.* 1986). Interestingly, this transition in chromatin structure requires both PHO4 and PHO2 (Fascher, Schmitz and Hörz 1990). *PHO5* is a tightly regulated gene with a very low basal level of expression. Under the conditions of my experiments, *MET16* appears to be less tightly regulated than *PHO5*, with a higher basal level of expression relative the induced level. If chromatin structure of a promoter region is a good indicator of gene activity, then the chromatin structure of the repressed and derepressed

MET16 promoter is consistent with *MET16* being less tightly regulated than *PHO5*. Under repressing conditions, the major *PHO5* UAS element is flanked by specifically positioned nucleosomes (Almer and Hörz 1986), while at the *MET16* promoter the data might be interpreted to mean that the region immediately downstream of the CDEI site is free of nucleosomes. Upon derepression, two nucleosomes on either side of the *PHO5* UAS element are lost, while the at the *MET16* promoter the changes observed support at most the loss of only one additional nucleosome. Thus, the structural differences observed between these two gene promoters correlate with their differences in regulation. Although the significance is unclear, the *PHO5* promoter contains twice the number of CDEI-like elements (PHO4 binding sites) and loses twice as many nucleosomes upon derepression as that proposed for *MET16*.

The regulatory mechanism governing expression of *MET16* is reminiscent of the mechanism which operates at the *HIS4* promoter. Devlin *et al.* (1991) have shown that a RAP1 binding site is required for BAS1/BAS2-dependent (basal control) and GCN4-dependent transcriptional activation of the *HIS4* gene. The RAP1 binding site partially overlaps a high affinity GCN4 binding site and appears to increase the sensitivity of the surrounding region to micrococcal nuclease. Since binding sites for BAS1, BAS2 and GCN4 fall within this region, it has been suggested that RAP1 maintains the accessibility of these factors for their binding sites within chromatin. At *MET16*, CP1 is also required for the normal operation of two activation mechanisms, appears to bind in close proximity to other factors, and increases the sensitivity of the *MET16* promoter to micrococcal nuclease.

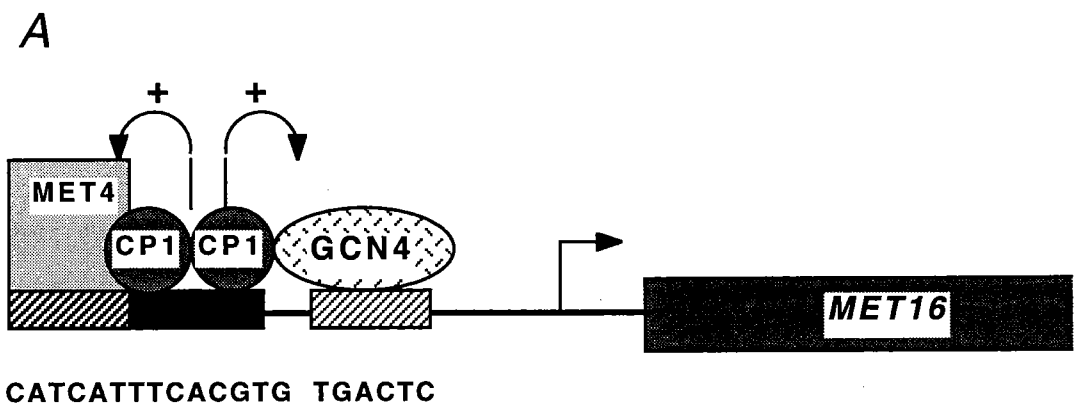
It is possible that all GRFs function by arranging chromatin in an "active" configuration. As discussed above, RAP1 increases the sensitivity of the *HIS4* promoter to nuclease attack, perhaps reflecting a repulsive effect on nearby nucleosomes. REB1 also affects chromatin structure, and at the GAL1-GAL10 intergenic region creates a ~230 bp nucleosome-free region, encompassing the UAS_G element, flanked on either side by arrays of positioned nucleosomes (Fedor, Lue and Kornberg 1988). However, it is not clear whether this effect has any functional significance since in synthetic constructs, GAL4 can efficiently activate expression in the absence of a RAP1 binding site (Chasman *et al.* 1990). The effect CP1 has on chromatin structure appears distinct from that of REB1. Most nucleosomes at the *MET16* promoter remain positioned in the *cep1* strain, and the ordered chromatin structure of yeast centromeres (Bloom and Carbon 1982) is only slightly affected (but not disrupted) by the *cep1* mutation. (Mellor *et al.* 1990). Thus, if CP1 functions by altering chromatin structure, it probably does so by a mechanism distinct from that of REB1.

One important question concerning GRFs is how they function in such diverse processes. Are they truly multifunctional or does each GRF possess a single activity required for different processes? Possibly, GRFs assist other DNA-binding factors solely by modulating chromatin structure. For instance CP1 might alter chromatin structure at centromeres and promoters to allow kinetochore components and transcription factors access to their binding sites. However, structure/function analysis of RAP1 and CP1 has indicated that these proteins possess multiple functions. Single missense mutations within the C-terminal region of RAP1 affect RAP1's ability to repress transcription of

the silent mating type locus *HMR*, without affecting RAP1's ability to activate transcription of essential genes (Sussel and Shore 1991). Furthermore RAP1 has been shown to form a complex with the transcriptional activator GCR1 to regulate expression of genes encoding glycolytic enzymes and translational components (Tornow *et al.* 1993) It is unlikely that RAP1 functions at *HMR* in a complex with a transcriptional activator, and thus RAP1 appears to function differently at these two genetic loci. Random mutagenesis of *CEP1* has also uncovered separable functions. Mutations within or just downstream of the b-HLH domain generated mutants disabled separately for the chromosome segregation function or the methionine biosynthetic function (Foreman and Davis 1993). It is still possible that CP1 actually does possess a single activity required for chromosome segregation and transcription and that these mutations simply block the ability of CP1 to provide that function at a subset of its binding sites. On the other hand CP1, like RAP1, might form complexes with other factors which confer upon CP1 a unique activity.

In summary, CP1 is required for transcription activation of *MET16* in response to methionine limitation, mediates it effect through the upstream CDEI site, and modulates the chromatin structure of the *MET16* promoter. CP1 is also required at the *MET16* promoter for an optimal general control response and at the *MET25* promoter for normal methionine-responsive regulation. It is presently not clear how CP1 functions at promoters but one model consistent with all the data is that CP1 recruits MET4 and GCN4 to promoters (Figure 12). This might be achieved by altering chromatin structure or possibly through direct protein-protein interactions with these factors as seen for RAP1.

FIGURE 12.—Two possible models to explain how CP1 functions at the *MET16* promoter. (A) CP1 recruits other transacting factors to the *MET16* promoter via direct protein-protein interactions with these factors. Model A predicts that CP1 makes direct contacts with the transactivators MET4 and GCN4. However GCN4 and MET4 still engage in protein-DNA interactions with important promoter elements. (B) CP1 recruits other transacting factors to the *MET16* promoter by modulating chromatin structure. In the model shown CP1 inhibits the formation of a nucleosome over the transcriptional initiation site thereby allowing basal factors to bind DNA. However, other effects on chromatin structure are possible. The CDEI site is indicated by the black rectangle, the GCN4-binding site by the rectangle with diagonal hatch marks, and the *MET16* coding region by the thick shaded rectangle. The sequences of important promoter elements are shown beneath the corresponding binding sites. Note that sequences to which MET4 binds are not known. In this figure MET4 is envisioned to bind immediately adjacent to the CDEI site.


A
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MET4
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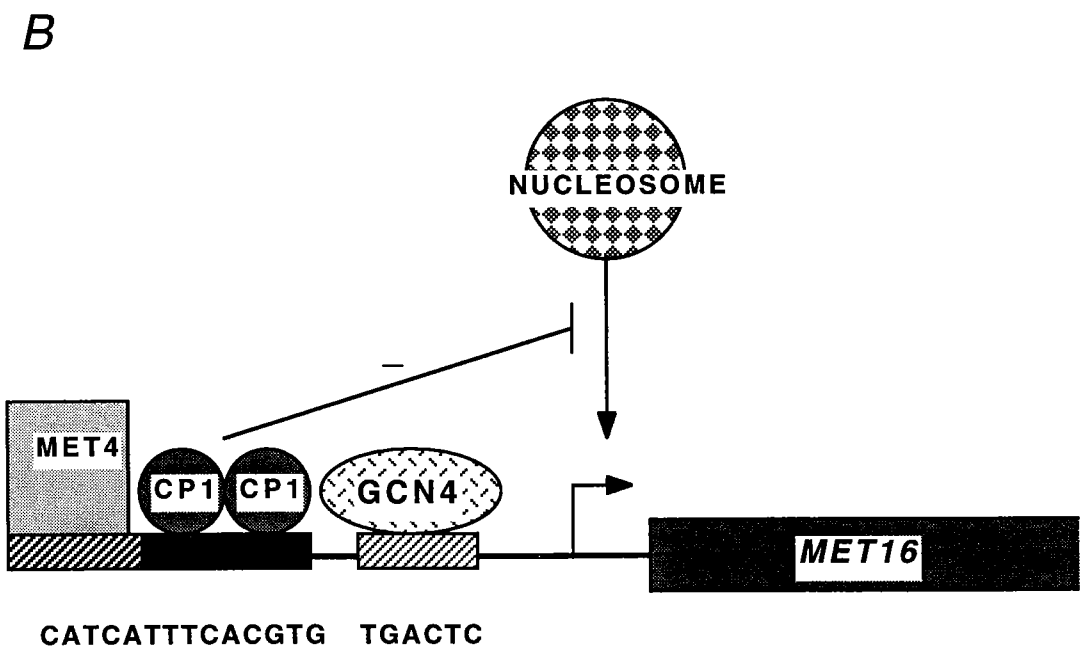
CP1
GCN4
MET16
CATCATTTCACGTG TGACTC


B
NUCLEOSOME
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MET4
CP1
CP1
GCN4
MET16
CATCATTTCACGTG TGACTC

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